NOTES ON THE DISTRIBUTION OF PIGMENT GRANULES IN THE RETINAL PIGMENT EPITHELIUM OF LIGHT- AND DARK-ADAPTED *RANA TEMPORARIA*, AND ON THE NATURE OF THE EYE PIGMENT IN THE FROG AND THE OX

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(With Two Text-figures)

UT two years ago, the writer made a study of the retinal pigment epithelium of ty-two English frogs which had been subjected to various experimental condi- and found that the dispositions of the pigment granules could not be explained uately in terms of the oft-described migration which is believed to take place ng light- and dark-adaptation of the eye. The results were anomalous, in so far e amount of pigment present under certain conditions seemed to be greater at other times.

Since these observations were first made, the writer has shown (1941) that frogs h have been kept for a period on equally illuminated dark or light backgrounds more significant changes than the mere dispersal or aggregation of melanin ules in the dermal melanophores, namely, the synthesis or the degradation of pigment. The disclosure of this fact has induced the writer to re-examine the a sections, and to present here extreme observations which tend to show, if they ot absolutely prove, that the pigment present in the retinal pigment epithelium es in amount under certain conditions of light and darkness.

Another aim of this work is to compare the pigments obtained from the eye and of the frog and the eye of the ox, using spectrophotometric data as the basis of parison.

MATERIALS AND METHODS

Of the twenty-two adult *Rana temporaria* used in this work, two were selected fifty individuals because they were the duskiest and the most pallid respectively, e were typically light-adapted, five were kept in total darkness at 15° C. for ods ranging from 1 hr. to 5 days, five were kept in darkness for periods of days and subsequently treated with the light of a carbon arc or of an ultra-violet ,* two were treated with post-pituitary extract and then dark- or light-adapted,

* This Hanovia lamp was obtained by means of a grant from the Dixon Fund.

two were completely hypophysectomized, and three were subjected to removal of the anterior lobe only of the pituitary complex. The whole series of experiments provided for study retinas which had received treatment calculated to provide various distributions in the granules of the pigment epithelium.

The following procedure was adopted in excision and fixation of the eye. The frog was pithed and, still in dim light, the eyes were speedily excised and placed in Bouin's fluid. After a few moments of superficial fixation, the eye was dissected expeditiously, the cornea, iris, and lens being removed in turn. Fresh fixative was then pipetted into the retinal cup, which was submerged in Bouin's fluid for at least 24 hr. The usual technique was followed in preparing the dissected eyes for sectioning in paraffin wax and both eyes were sectioned at a constant thickness of 8 μ. One eye of each individual was cut vertically and at right angles to the aperture, the other was cut vertically but tangentially to the aperture. The median-most sections of the first set show a continuous extent of retina from the dorsal rim to the ventral rim across the fundus, while sections of the second set show a complete circlet of retina at various points increasingly distant from the fundus. The sections were stained in Delafield's or Ehrlich's haematoxylin and counterstained lightly in Chromotrop 2R. They were dehydrated, cleared, and mounted in the usual manner.

The pigment of the eye was collected from three batches of three frogs, and from two separately treated ox eyes, in the following manner. Cornea, iris and lens were excised and the vitreous humour was removed. The nervous and visual layers of the retina were greatly separated from the underlying pigment epithelium and choroid coat, which were next extracted together and agitated in warm 0·4% NaOH. The pigment of the eye does not enter solution as readily as does that of digested skin, but a few minutes of boiling in the alkali sufficed to produce a deep brown solution, the strength of which was adjusted so that all samples were of approximately equal depth of colour. Observations were made at several dilutions and the mean result was used. A random sample of pigment from the skins of very dark individuals was collected by methods previously described (Dawes, 1941) and this was studied at six dilutions, the mean result being compared with mean results obtained with the eye pigment. Spectrophotometric data were obtained by the use of a Duboscq colorimeter and the apparatus, colour standards and filters used were the same as those employed in the previous work (Dawes, 1941). Curves of log density/wavelength varied slightly, being slightly steeper for high than for low dilutions, suggesting some scattering as well as true absorption of light. But the general results lack ambiguity and clear conclusions can be drawn.

RESULTS

No attempt will be made to describe fully the distribution of pigment granules in all the retina sections examined. A selection of extreme data will suffice to support the writer's main thesis, which is the quantitative variability of the eye pigment under various conditions of light and of darkness. It is emphasized, however, that statements concerning a particular retina have been checked against the appearance

ther retinas where possible, so that the results are not based upon observations single retina except where this is specified as being the case.

The duskiest and most pallid individuals of fifty (E 1 and E 4 respectively) were for two days in dimly lit vivarium at 15° C. on a background which was neither k nor white. The light conditions were such as favour partial light-adaptation of eye, and the granules of the retinal pigment epithelium show the degree of ersion which would be expected from consideration of the treatment. Despite difference in skin pigmentation of these two individuals, implying at least twice uch pigment in the one case as in the other, the retinas were found to be almost tical as regards the number and distribution of pigment granules. It is true to that the retinas might be interchanged without affecting the general observa- s made. At the fundus of the retina, in each case, relatively few ovoid or spherical ules occur in the cell body, while the cell processes contain numerous rod-like ules, which are aggregated most densely about the basal parts of the visual ents, i.e. the parts nearer the external limiting membrane (Fig. 1 A). A few e or less isolated granules lie adjacent to the lentiform bodies of the rods, and the entration of granules steadily diminishes towards the basal parts of the cell esses adjacent to the free terminations of the rods.

At the rim of the retina differences are seen which are common to both these viduals. The bodies of the pigment cells are so crowded with ovoid granules as ppear dense black at the bases of the cell processes (Fig. 1 B). The cell processes nselves contain numerous rod-like granules, however, and the excess of granules e cell bodies must be far greater than could be accounted for by the somewhat ter cell processes in this narrower region of the retina. The amount of pigment he cell bodies appears steadily to increase in cells progressively more distant n the fundus and progressively nearer the rim of the retina.

Two conclusions can be drawn from these observations: (*a*) the amount of ment present in the retinal pigment epithelium does not vary in accordance with ation in the amount of melanin present in the skin, and (*b*) the amount of ment in the retinal pigment epithelial cells is probably greater near the rim than the fundus of the retina. The first conclusion was reached after study of only individuals, but these undoubtedly showed extremes of skin pigmentation. The nd conclusion was tested and found to hold good for every dark-adapted and ially light-adapted retina examined.

One individual (E 5) was taken out of diffuse light and kept in darkness at 15° C. two hours. According to Arey (1916), who summarized his own observations those of other workers, the pigment of the dark-adapted frog retina is massed in bodies of the pigment epithelium cells at 14–19° C. but scattered in the basal s of the cell processes at 0–14° C., and at 19–33° C. Detwiler & Lewis (1926) rmined slightly different temperature effects, observing a massing of granules n as is characteristic of full dark-adaptation after 1 hr. at 18–23° C. and after . at 15 and 17° C. The writer's individual, E 5, showed numerous pigment ules in the basal portions of the cell processes and fairly dense concentrations anules in the cell bodies of the pigment epithelial cells, suggesting that the lower

extremity of the temperature range at which dark-adaptation is incomplete in darkness is a lower temperature than Arey and even Detwiler & Lewis found in their experiments.

The writer was prompted to ascertain the result of keeping animals for much longer periods in darkness, and four individuals (E7, E9, E12 and E13) were subjected to conditions of darkness for 2, 3, 4 and 4 days respectively. It is interesting to note that even after 4 days in darkness at approximately 15° C. some pigment granules were found in the processes of the pigment cells (Fig. 1 D represents the condition after 2 days). There is no indication, however, of diminution in the amount

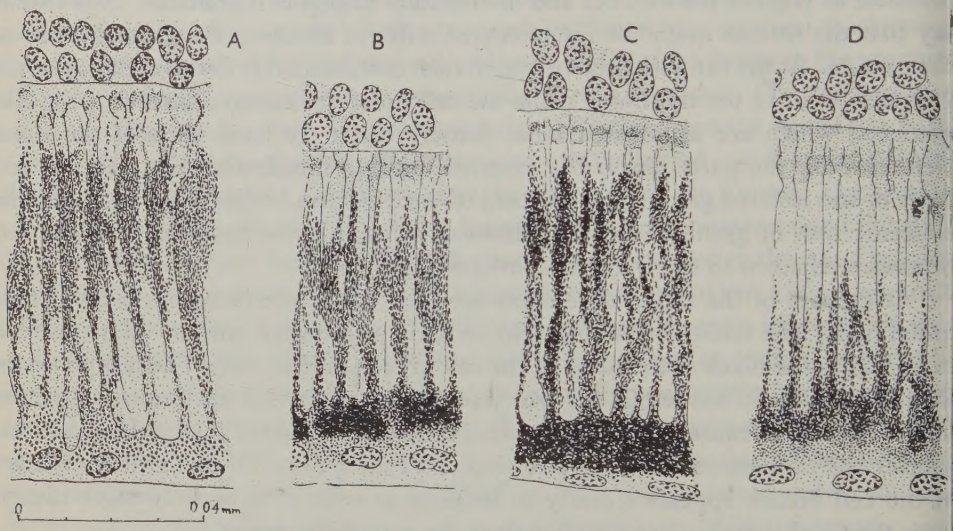

Fig. 1. Vertical sections of portions of the retinas of *Rana temporaria*. The region represented extends from the external nuclear layer (upper parts of figures) to the base of the pigment epithelium (lower parts). A, fundus of incompletely light-adapted retina of E1; B, rim of retina of E1; C, fundus of retina of E14 (light-adapted with ultra-violet radiations); D, fundus of E7 (animal kept in dark for 2 days at 15° C.). C shows anomalous condition; dense accumulation of granules in both the processes and the bodies of the pigment cells.

of pigment present, that is to say, the intensity of pigmentation after 3 or 4 days in darkness is apparently the same as that after only 2 hr. in darkness. These four individuals provide a contrast with the partially light-adapted individuals (E1 and E4), however, in showing a greater concentration of granules in the cell bodies and fewer granules in the cell processes. This is a characteristic difference which has been described often in the literature bearing on light- and dark-adaptation of the eye.

The greatest contrast with the above-mentioned retinas was provided by the retinas of individuals which had been kept for various periods, up to 5 days, in darkness and then treated for short intervals with the radiations of a mercury vapour lamp or of a carbon arc (water-screened). The individuals E14, E15 and E16 were subjected to the radiations of a lamp situated at a distance of 18 in. for 10 min., 35, 60 and 75 min. respectively in dim light being allowed for development

e effect. Any one of these retinas shows a distribution of pigment granules which ntrasts strongly with that previously described, but granules are seen to lie arer to the external limiting membrane when a longer period is allowed after adiation. In E14 (Fig. 1C), pigment granules extend to the lentiform body of the ds, which must mean to the limits of the cell processes. The granules in the cell ocesses are far more numerous than in the case of E1 and E4, and are quite as merous as in typical light-adaptation. At the same time, it is important to observe, nse aggregations of granules occur in the cell bodies as well. This is the anomalous cture of pigmentation in the retinal pigment epithelium. The intensity of pig- entation (i.e. the concentration of granules) in the cell processes is quite as great as typical light-adaptation, while that in the cell bodies is even greater than in pical dark-adaptation. The bodies of the epithelial cells on the side of the nucleus stant from the choroid are so densely crowded with granules as to appear solid ack. This condition is intensified in E15 and E16, and in order to counter any ggestion that the effect is pathological, it must be stressed that the appearance of the layers of the retina and particularly the visual elements was perfectly normal, cept in this matter of pigmentation. It is impossible to avoid drawing the con- usion, cautious as one must be in the absence of definite quantitative data, that the mount of pigment is greatly enhanced under the conditions specified.

Other experiments need be mentioned but can be described briefly. One dividual (E6) was hypophysectomized and henceforth remained excessively pale, en on a black background. It was kept in a black vessel, illuminated by diffuse aylight only, for 5 weeks. The retina of this individual is similar as regards pig- entation to that of a normal animal which has been subjected to conditions of ther dim illumination. Numerous granules are seen in the bodies of the epithelial lls, the central parts of which are almost solid black. Some granules occur also in e basal halves of the cell processes. The amount of pigment in these cells and their ocesses appears to be quite as considerable as in the normal animal; certainly, no arked diminution in the amount occurs as a result of hypophysectomy. This latter sult was confirmed in a second hypophysectomized individual (E17), which mained very pale during the 4 weeks it was kept on a black background in diffuse ght. This individual finally received light from a water-screened carbon arc set 8 in. away for 15 min., after which period 10 min. were permitted for development f the light effect in dimly lit surroundings. Numerous granules occur in the cell odies and cell processes of the retinal pigment epithelium of this individual. xcision of the pituitary complex does not interfere with the normal effect of light n the pigment epithelium. It might also be mentioned here that in three individuals E18, E19 and E20) the removal of the anterior lobe only of the pituitary complex roduced similar negative results.

One other observation which might prove to be important concerns the length f the visual elements in hypophysectomized individuals. The rods in E6 and E17 easure only 0·052 mm. in length, while those of E1 and E4 are 0·085 mm. long.*

* Krause (quoted by Gaupp) states that the rods of the frog are 0·081 mm. long. The writer's easurements extend from the external limiting membrane to the free terminations of the rods.

The rods of these hypophysectomized individuals thus appear to be significantly shorter than those of the normal animal.

Two individuals received subcutaneous injections of 0·5 c.c. post-pituitary extract, which raised the melanophore index from 2·5 to 4·5 in 1 hr. irrespective of subsequent treatment. One of these (E2) was placed in darkness at 15° C. for 1 hr.,

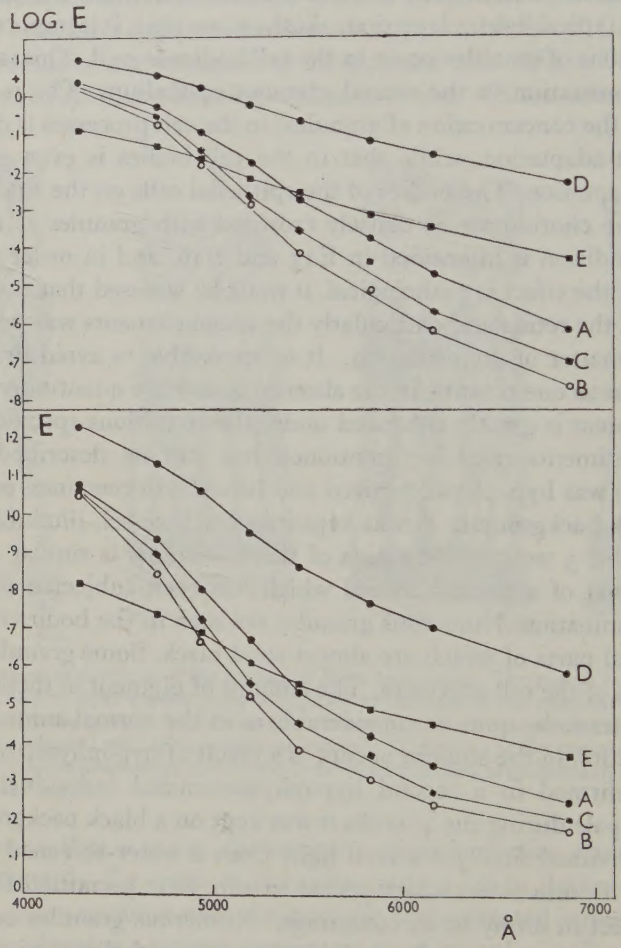

Fig. 2. Curves of wave-length/density (below) and wave-length/log density (above) for the pigmen extracted from the eye of *Rana temporaria* (A), the eye of the ox (B), and the digested skin of *Ran temporaria* (C). Other curves prepared from data compiled by Dawes, 1941; D, pigment from ski of mean dark and medium-dark animal; E, pigment from skin of mean animal irrespective of the ton of the skin.

while the other (E3) was placed in bright sunlight for the same length of time. I the case of the former individual, much pigment occurs in the cell bodies of th pigment epithelium and some pigment in the basal parts of the cell processes; in th case of the latter individual, less pigment occurs in the cell bodies but more occu in the cell processes, the granules extending almost to the external limiting mem

ane. The difference between the retinas of the two individuals is precisely that inced in normal dark- and light-adaptation of the eye. These results are presented ntatively, and though it would be unwise to base an important conclusion upon such nty evidence, it would seem reasonable to suggest that while adrenaline produces apparent scattering of pigment granules in the pigment epithelium of the dark-apted eye (Fujita, 1912; Bigney, 1919; Nakamura & Miyake, 1922; Ducret & go, 1931), pituitrin is without apparent effect upon both the dark- and the light-apted retina. This is not in complete agreement with the findings of Jores & esar (1935), who found the melanophore hormone to be without effect upon the ht-adapted eye but who believed they recognized an accelerated movement of gment into the "dark" position during dark-adaptation under the influence of this rmone.

Photospectrometric data concerning the pigment of the eye and skin of the frog d the eye of the ox are presented in Fig. 2 in the form of plots of wave-length/ nsity and wave-length/log density. The curves for the pigment of the frog's eye) and that of the ox's eye (B) are of similar slope, and the curve for melanin from e skins of dark frogs (C) is of intermediate slope. The three curves are sufficiently iform to warrant the conclusion that no significant difference exists between the ree pigments. Curves derived from the writer's previous data (1941) are also own in the figure and are seen to be less steep than the newly prepared curves. he steepness of the curves A, B and C serve to strengthen the writer's previous atement to the effect that mammalian and amphibian melanin are closely similar not identical and permit us to include in this generalization the pigment of the eye m one member of each class.

DISCUSSION

The main observations which have been made concerning the distribution of gment granules in the retinal pigment epithelium of frogs following treatment with ght or darkness can be summarized as three conditions: (*a*) in which granules are gregated only in the bodies of the cells and the basal parts of the cell processes,) in which granules are aggregated in the processes of the cell while few or none cur in the bodies, and (*c*) in which dense aggregations of granules occur in both e processes and the bodies of the cells. The first two of these conditions fully present what is generally believed to be a migration of pigment granules into or ut of the processes of the cells under the influence of light or darkness. The third ondition is anomalous in that it portrays more than can be explained in terms of the igration of granules, namely a crowding of granules such as is characteristic of ght-adaptation in the cell processes and of dark-adaptation in the bodies of the lls. This condition of increased pigmentation was produced by the radiations of a ercury-vapour lamp and it is not the accompaniment of injury to or pathological ange in the retina. Accordingly, there must be a rational explanation of it.

In attempting to explain the anomaly, the writer is prompted to observe that e idea of pigment migration is based upon a vast amount of evidence but

upon evidence of a circumstantial nature. So far as the writer is aware, actual movement of granules in the vertebrate retinal pigment epithelium has not been witnessed. The idea of movement has arisen in explanation of separate pictures of the distribution of granules in dark- and light-adapted retinas. In short, movement is assumed to take place. An alternative explanation could be found; for instance, the separate dispositions of granules in dark- and light-adapted retinas might conceivably be due to concomitant processes of destruction and formation of pigment granules in different parts of the pigment cell. Or, a process of bleaching of pigment in one part of the cell to the accompaniment of fresh pigmentation in another. Such bleaching is well known to be readily accomplished *in vitro*. According to Miescher (1923), the shape of the pigment granules is due to an albuminous ground substance upon which the actual pigment is deposited by a process akin to adsorption, an arrangement which might conceivably facilitate such processes of addition and subtraction of pigment. In this view, the anomalous aggregation of granules in both the cell processes and the cell bodies of the pigment cells could be explained on the assumption that pigment formation is enhanced or the bleaching of pigment impeded, or that both these processes occur.

Several considerations indicate that the idea of pigment migration is fallacious. The body of the retinal pigment cell contains small rounded, amorphous granules and also crystalline needles or rods of pigment, while the processes contain needles or rods only (Arey, 1932). The absence of rounded granules in the cell processes is explained as being due to the greater mobility of the rod-like granules, which are swept along in a protoplasmic stream (assumed) more readily than are the rounded granules. It would not be unreasonable to suppose, however, that if such streaming of protoplasm occurs from the base of the cell into the processes it would carry with it some rounded granules, since these are mixed with rod-like granules in the basal part of the cell. Yet such is never found to be the case. The true explanation of the fact that only rod-like granules occur in the cell processes may well be that they develop or acquire their pigmentation in the situation in which they are found. Migration is even more difficult to comprehend when other granules of a rather ambiguous character are taken into account. Thus, Arey (1932, p. 747) draws attention to "the full retreat of pigment in certain dark-adapted retinas while the tapetal guanin still occupies a more distal position". There follows a reference to a figure showing a section of part of the retina of *Ameiurus* in which pigment granules occupy a basal and "pseudo-guanin" granules a distal position in the pigment cells. How far such granules are actually composed of guanin and to what extent they might otherwise be regarded as derived from or antecedent to pigment granules is doubtful. Ovio (1927) states (p. 437), "Dans l'œil-obscurité, la guanine et le pigment se retirent—. Mais le pigment se retirant plus que la guanine, une grand masse de celle-ci reste découverte", and "Dans l'œil-obscurité de l'abramis, s'accomplirait donc partiellement, au niveau du tapis, une migration en sens inverse de pigment et de guanine. Le pigment se retire complètement, la guanine avance en partie." Such differential "movements" are inexplicable in terms of protoplasmic streaming, just as is the appearance of superlative amounts of pigment massed at the

ame time during light-adaptation in both the body and the processes of the retinal igment cell. With all due deference to the sustained observations of very numerous orkers, the writer suggests that whether or not pigment migration actually occurs, rocesses of addition and subtraction of pigment materials are probably at work uring light- and dark-adaptation.

The true function of the retinal pigment has not been determined. That it sorbs light is evident, but what is accomplished by this absorption of energy is nknown. According to Parker (1932), who reviewed this subject, the early workers garded the pigment as a means whereby light energy is transformed into that form energy which is appropriate for stimulating the visual apparatus, while more cent workers agree that absorption of light is a means of eliminating light which ould otherwise disturb the sharpness of the visual image or which would provide overpowering stimulus. The pigment epithelium may play a much greater part in tinal function than merely to provide a light screen to the visual elements. Many atures of the epithelium point to polarized secretory activity (its proximity to the ch capillary network of the choroid, the basal position in the cells of the nuclei and e distal position of various granules, the arrangement of numerous processes mbracing the secreted materials which form the outer segments of the visual ements), and such activity has been attributed to it (see Parker, 1932), but rarely. ameron (1905) observed that the processes of the pigment cells develop in frog rvae simultaneously with the rods and cones, and postulated a "positive chemo-xis" between the two sets of elements which is evinced before light exerts any fluence. He claims that the nucleus contains a substance with the power to digest nd absorb pigment and expresses his belief that the developing rods owe their owth to this process. Finally, he asserts that this behaviour is simply an index of vents later taking place, the pigment cells producing pigment which is used in the anufacture of visual purple. Such a view may be erroneous (it has been neglected y many workers and criticized by a few) and yet hold more than a fragment of uth. Luna (1911) also claimed that pigment granules are consumed progressively nder the influence of light.

That chemical changes occur in the retina under conditions of light and darkness well known. Birnbacher (1894) showed that the visual elements possess greater finity for acid dyes after dark-adaptation, and this result has been confirmed odato, 1895; Majima, 1925). Tansley (1935) observed in the retinas of frog and t differential staining effects which seemed to indicate that the outer segments of ne visual elements become less acid during dark-adaptation. If staining reactions re deemed unreliable criteria, it can still be noted that Mori (1921) recorded a iminution in the glycogen content of the retinas of frog, rabbit and guinea-pig nder the influence of light, and that Lange & Simon (1922) noted an increase in the hosphoric acid content of the retina under similar conditions. More direct evidence f the intervention of the pigment epithelium in such chemical processes is shown y the fact that visual purple fails to regenerate in darkness when the epithelium has een loosened from the visual elements (Arey, 1932; Parker, 1932). Regeneration ccurs, however, when contact is restored, and it is hastened by pilocarpine and

muscarine, apparently as a result of increased secretory activity on the part of the epithelial cells (Arey, 1932).

Possible correlation between the photochemical activities of the retina and endocrine activity has received little attention. Kropp (1929) has shown, however, that extracts of the eyes of dark-adapted tadpoles or *Fundulus* will cause "expansion" of the dermal melanophores of light-adapted animals. Vancea (1936), who reviewed some phases of endocrinology in connexion with diseases of the eye, refers to evidence of endocrine activity of "actino-receptive" organs, including the eyes. Enucleation of the eyes may lead to hypertrophy of the upper jaw, atrophy of the testes, and changes in blood chemistry. It may be pure coincidence that the retina contains "red" and "green" visual elements and that the pigment "hallachrome" (now believed to be identical with the red intermediate product which is formed during melanogenesis) is red on the acid and green on the alkaline side of *p*H 8·3 and is capable of reversible oxidation and reduction (Lederer, 1940). But it is conceivable that the pigment epithelium is indispensable to essential chemical syntheses which occur in the visual elements of the retina and that polarized changes in its cells are more indicative of such syntheses than they are of protoplasmic movements.

SUMMARY

The retinas of twenty-two adult *Rana temporaria* were examined following varied experimental treatment. The disposition of pigment granules in the pigment epithelium cannot invariably be explained in terms of migration. Individuals which were subjected to the radiations from a mercury-vapour lamp showed dense aggregates of pigment granules in both the bodies and the processes of the pigment cells. The possible significance of this observation is discussed.

The amount of pigment in the epithelium does not appear to vary in accordance with the amount of melanin contained in the skin. The amount of pigment appears to be greater, however, in pigment cells near the rim of the retina than in cells at the fundus of the retina. Hypophysectomy of 4 or 5 weeks' standing is without effect on the amount of pigment present in the pigment epithelium or on the disposition of granules in light- and dark-adapted retinas. Subcutaneous injection of post-pituitary extract is likewise without effect on the disposition of granules under conditions of both light and darkness.

Pigments extracted from the choroid and retina of the frog and the ox have been compared with one another and with the pigment extracted from the digested skin of the frog. Spectrophotometric data indicate no essential difference between these pigments.

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OSMOTIC BEHAVIOUR OF THE FAIRY SHRIMP *CHIROCEPHALUS DIAPHANUS* PRÉVOST

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(With One Text-figure)

Nothing is known of the osmotic concentration of the blood of fresh-water phyllopods. In the cladoceran *Daphnia magna*, Fritzsche (1917) obtained freezing-point depressions for blood varying between $-0{\cdot}2$ and $-0{\cdot}67°$ C. Krogh (1939) did some preliminary experiments on *Branchipus* (? *grubii*) and *Apus* (*Lepidurus*) *productus* and found that these animals can be kept alive without food only for a day or two, whether they are in tap water, distilled water or in Ringer/100. Regular loss of chloride occurred in all experiments, which indicated the absence of active regulation and the necessity of food to make good the constant loss of salts. *Artemia*, which is closely related to *Branchipus*, inhabits concentrated salines up to about 35% salt and shows highly developed powers of regulation involving ability to maintain hypotonicity to the external medium. By Barger's method, Medwedewa (1927) and Kuenen (1939) found that the osmotic pressure of its blood varied only between 1·2 and 2·6% NaCl for a change in the external medium from 4·7 to 17·7% NaCl. *Chirocephalus*, which belongs to the same tribe of phyllopods as *Artemia* and *Branchipus*, is an essentially fresh-water form, though by frequent drying and flooding the pools in which it lives may accumulate slight quantities of salt. Weldon (1909) found them alive in an aquarium where, by evaporation, the chloride content of water had risen up to 0·019% NaCl. The following experiments were performed in order to study the osmotic properties of *Chirocephalus* when a limited supply was available in the summer of 1940.

The material was kindly collected for me from pools of fresh water on Roborough Down, near Plymouth, by Mr G. M. Spooner to whom my grateful thanks are due. A subsequent culture was made in the laboratory from mud taken from the bottom of jars in which the shrimps had lived and died. The osmotic pressure was determined correct to $\pm 0{\cdot}003$% NaCl by Baldes's micromodification of the Hill vapour pressure method; details of procedure adopted are given in another paper (Panikkar, 1941). For removing the blood, each animal was first placed on a dry filter paper to remove the adhering water and later, on a slide, under a binocular microscope, the blood being withdrawn from the dorsal blood vessel with a specially prepared glass cannula. The blood was transferred to the thermocouples almost immediately after withdrawal; one *Chirocephalus* provided enough blood for two estimations. The mean value for each sample is represented by each point on the graph (Fig. 1).

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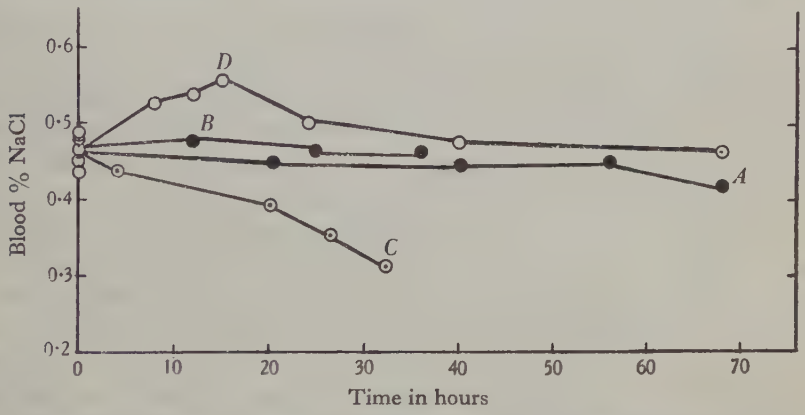
The blood showed an osmotic pressure equivalent to 0·44 to about 0·5 % NaCl an external medium (natural habitat) of 0·002 % NaCl. These values were tained a day after collection and it was later found that there was little change en after the animals had been living in the laboratory for some days in the same ter and mud brought from Roborough Down. There was no appreciable difference tween the osmotic concentrations of males and egg-bearing females.

When *Chirocephalus* was kept without food in filtered tap water, there was no preciable decline in osmotic pressure, but the animals were not able to live for g in this medium (Fig. 1 *A*, *B*). In most of the experiments all individuals died thin the first 50 hr.; in one experiment, however, two specimens lived for 3 days, er which they became inactive. Osmotic pressure of one of them was determined this condition and showed a very slight fall from the normal. The other specimen ed within a few hours.



ig. 1. Changes in the osmotic concentration of the blood of *Chirocephalus* in tap water (*A* and *B*, o series of experiments), glass distilled water (*C*), and very dilute hypotonic sea water of 0·351 % aCl (*D*). Abscissae, time in hours; Ordinates, osmotic pressure of blood in % NaCl. Range of ormal osmotic pressure expressed at zero time.

In glass-distilled water, *Chirocephalus* could live only for a comparatively short eriod. Most of the experimental specimens died within 36 hr. The osmotic ressure of these animals gradually fell from the normal (0·44–0·5 % NaCl) to about ·3 % NaCl, which is probably the minimum dilution of blood which it could urvive (Fig. 1 *C*). Plymouth tap water contains a trace of chloride and this would ppear to be helpful to the animals in maintaining their normal concentration in ap water in the absence of food. It is interesting to note that two individuals, hich were in glass-distilled water for 32 hr. and had become inactive and sunk to ne bottom of the jar, slowly recovered when taken to tap water, though they died n the new medium after a lapse of a further 30 hr.

The above results suggest that *Chirocephalus* is able to assimilate ions from tap ater. To prove the occurrence of ion absorption conclusively, a batch of specimens as transferred to very dilute sea water having an osmotic pressure of 0·351 % aCl. Blood from experimental animals measured at intervals showed a rise in

value from the normal 0·4–0·5 to about 0·55% NaCl, within 15 hr. The osmotic pressure returned to normal afterwards as shown in the graph (*D*). The increase in value in this experiment cannot be due to osmotic phenomena since the external medium was definitely hypotonic, but can only be due to the active absorption of salts against the osmotic gradient. It would appear from the initial rise in osmotic pressure that the normal process of ion absorption was continued at the same rate for some time, even after transfer to a medium richer in salts than the former one and that the value was brought to normal by later regulation. All the animals in this medium died after 3 days.

When transferred directly to sea water from fresh water, the specimens died within an hour or two. They became inactive in less than half an hour, but recovered when immediately taken back to tap water. Acclimatization experiments with very dilute sea water of osmotic pressures 0·210 and 0·298% NaCl failed, since in both media all animals died within 3 days.

In all experiments where salt water was employed, a change in the appearance of the bracts became evident after some hours. In the normal animal the bract, which is the proximal exite of the thoracic limb and the only part of the limb without spines or setae, is transparent like the rest of the appendage. In salt water these structures slowly turn opaque; the higher the salt concentration of the medium the sooner this happens. This change is seen before the animal shows any sign of distress or inactivity. The bracts of *Chirocephalus* are homologous with the gill-sacs of Cladocera and are believed to be respiratory in function. The cuticle covering them is extremely thin as compared with that of other regions of the animal. In a paper published before the concept of ion absorption in animals was introduced by Koch and Krogh, Dejdar (1930) with the technique developed at Prague by Gicklhorn and Keller, investigated a number of branchiopods, including *Chirocephalus*; he showed the selective reaction of the bracts to vital staining with dyes and dilute solutions of silver salts and other substances. From the similarity in behaviour of the bracts and the dorsal organ (Nackenschild), especially during the early stages, Dejdar concluded that the latter is a respiratory structure that functions during larval life. Koch (1934) has explained these vital staining phenomena on the basis of active absorption of ions which is now well known from Krogh's work. I have obtained results similar to those of Dejdar by adding drops of millimolar silver nitrate to jars containing *Chirocephalus* in glass-distilled water. When the silver salt is reduced with 0·5% $KMnO_4$ in strong light, the bracts show the characteristic black appearance whereas the remaining parts of the appendage remain unaffected.

One may conclude from this fact that the site of the inward passage of ions (and possibly their outward passage in *Artemia*) is in the bracts. Whatever other functions they may have, the bracts would thus seem to have a role in the osmoregulation of branchiopods. If this conclusion is correct, it is likely that the dorsal organ has a similar function during the larval stages of many branchiopods and in the adults of forms like *Leptodora* which are without bracts but retain the dorsal organ.

DISCUSSION

The general behaviour of *Chirocephalus* appears from these experiments to be similar to that of *Branchipus* and *Lepidurus*, examined by Krogh (1939), in its ability to survive in media without food except for short periods. Fall in osmotic pressure of animals kept in glass-distilled water implies the continuous loss of chloride and this Krogh found in the two genera he examined. These three phyllopods differ from *Daphnia* and possibly other Cladocera which can live in pure distilled water for several days without food (Naumann, 1933). It is reasonable to assume that *Daphnia* is able to control loss of salts more efficiently than these phyllopods or that ion absorption is so efficient that the chloride lost is immediately reabsorbed.

In a recent note, Beadle & Cragg (1940) have questioned the universal importance of active ion absorption in the osmoregulation of fresh-water animals. They found that *Gammarus pulex* and *Asellus aquaticus* (both fresh-water forms) can live in distilled water for at least 8 days without food. While the fresh-water variety of *Gammarus duebeni* could live in distilled water for at least 4 days, the brackish water variety of the same species rapidly lost chloride and eventually died. Though active ion absorption does take place in these animals, they conclude that the essential part of the osmoregulatory mechanism is the power of retention of salts and not the power of absorbing salts. Salt retention may thus vary in closely related species and even in physiological races of the same species.

Judging from the results obtained with *Chirocephalus*, it appears to me that ion absorption plays a more important role in this animal than in fresh-water gammarids or even *Daphnia*. Only this could explain the longer period of its survival in tap water without serious fall in osmotic pressure, as compared with its behaviour in distilled water. Absorption of ions seems to be important at least in those fresh-water animals with feeble powers of salt retention. In animals where the loss of salts is small, uptake of salts under normal conditions is probably less significant. In the latter, not only the loss of salts, but also the entry of water into the animals, has to be minimized and, I think, the permeability of the integument is a vital factor. If the amount of water entering osmotically into the animal is small the loss of salts by way of the urine could also be brought to a minimum. It is worth mentioning that, as in euryhaline crustacea like *Carcinus* (*vide* Webb, 1940), ion absorption seems to play a significant role even in the homoiosmotic crustacean *Palaemonetes varians*, which can live in media varying from water that is nearly fresh to concentrated sea water equivalent to 5·0% NaCl (Panikkar, 1939, 1941). This prawn is unable to live in distilled water for more than a few hours, but lives for about a day in tap water. The period of survival without food could, however, be prolonged for many weeks if the chloride concentration of the medium is raised to about 0·010% NaCl. Successful adaptation of an animal to fresh water would depend upon its ability to maintain a favourable balance between the amount of salts lost into the medium and the amount absorbed against the osmotic gradient. It would seem that ability to survive for long periods without food in distilled water

and in inert media like glycerol would only be observed in animals where the loss of ions from the body is so small that the osmotic concentration of the blood is maintained well above the minimum threshold.

SUMMARY

The blood of *Chirocephalus* has a normal osmotic concentration equivalent to 0·44–0·5 % NaCl in an external medium of 0·002 % NaCl. The osmotic pressure falls rapidly when the animal is kept in glass-distilled water, but is fairly well maintained in tap water. There is an initial rise and a later return to normal even in hypotonic saline media, and this indicates active absorption of ions. The bracts are presumably the organs concerned in the salt absorption. The animal is unable to live in tap water or distilled water for more than 2–3 days without food and its general behaviour is thus markedly different from that of *Daphnia*.

I wish to thank the Director and the staff of the Plymouth Laboratory for their interest and helpful advice in this work.

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THE MECHANISMS OF HUMIDITY REACTIONS OF TERRESTRIAL ISOPODS

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(With One Text-figure and Seven Graphs)

INTRODUCTION

ECENT work by Gunn (1937) and Miller (1938) on land isopods clearly shows that ese animals are very sensitive to humidity gradients, aggregating in the areas of ghest humidity. The present series of experiments were carried out mainly on *orcellio scaber* Latr., to analyse the mechanisms of reactions whereby the animals e retained in the damper zones. Attempts were also made to see whether the haviour pattern, shown by Ullyott (1936) to be characteristic of the phobotactic actions of the planarian *Dendrocoelum lacteum*, is also exhibited in the humidity actions of the woodlouse. Further comparative experiments on *Oniscus asellus* Lin. d *Armadillidium vulgare* Latr. were undertaken to find whether there are any fferences in the hygrokinetic responses correlated with the natural habitats of the ree species, and how far the differences in behaviour were influenced by the rate evaporation of water from the woodlice.

METHODS

The apparatus initially used was a rectangular dish 3 in. deep, covered by a neet of glass with a perforation in the centre for the insertion of the animal. platform of perforated zinc $2\frac{1}{2}$ in. above the glass floor was supported on glass ods. The required humidities were obtained by placing dishes with sulphuric acid f known specific gravity underneath the zinc floor on either side of the dish. dney paper hygrometers were used for measurement of humidity. The range of ne gradient obtained was large, namely, from 20 to 90% R.H. However, this pparatus was found to be unsuitable for work with *Porcellio scaber*, which is trongly thigmokinetic and hence tends to keep very close to the sides of the dish hen moving, pressing one of the antennae against the glass wall. Moreover, this nimal showed a tendency to remain motionless at the corners of the dish. It was nteresting to note that when *P. scaber* was placed in constant humidities in this ype of apparatus, its thigmokinetic response increased with the rise of relative umidity. There appeared to be a balance between thigmokinetic and hygrokinetic ehaviour, and further experiments were undertaken to elucidate this point (see ; 118).

The second type of apparatus tried was the 'alternative chamber' described by Gunn & Kennedy (1936). This, in spite of being free from corners, again proved unsuitable, since the animals instead of moving about freely on the zinc platform tended to keep to the sides of the glass dish.

A tubular apparatus had therefore to be chosen, since it presents an equal surface of stimulation throughout its whole length to the strongly thigmokinetic animals. A modification of Gunn's circular humidity gradient apparatus was tried, and proved to be suitable. The apparatus used (Fig. 1) consists of a wide glass tube $1\frac{1}{2}$ in. in diameter and $58\frac{1}{2}$ in. long, bent into an oval. A circular tube would have been preferable, but this could not be obtained owing to difficulty of construction.

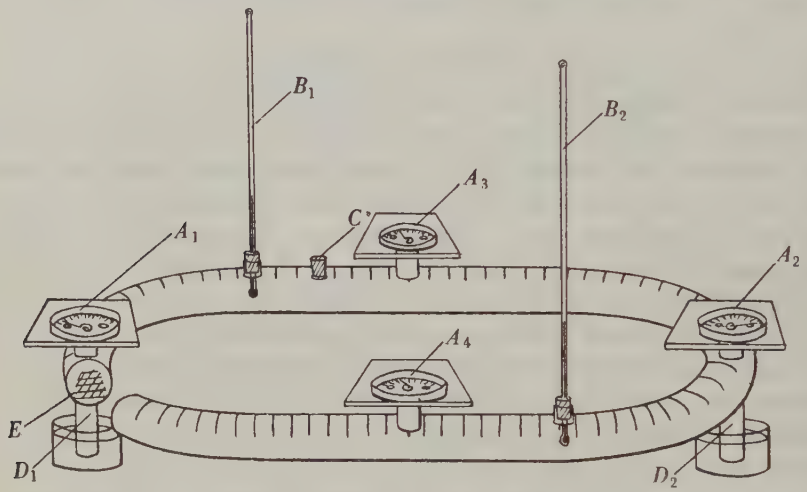


Fig. 1. Tubular humidity gradient apparatus for testing locomotory reactions of thigmokinetic animals. A_1, A_2, A_3, A_4, funnels with hygrometers; B_1, B_2, thermometers; C, opening for insertion of the animal; D_1, D_2, tubes dipping into sulphuric acid; E, perforated zinc platform.

However, the rounded corners of this apparatus did not appear to impede the progress of the animals or to alter their behaviour in any way. Externally the tube is divided into inches by thin strips of adhesive paper, and the inches are marked in white ink. At the two ends of the apparatus, and in the middle of each tube connecting the two ends are glass funnels A_1, A_2, A_3, A_4, designed to hold Edney paper hygrometers. Holes are bored in the hygrometers to ensure that the readings taken in the funnels are identical with those in the tube. The funnels A_1, A_2, A_3, A_4 are covered by small glass plates. Tubes B_1 and B_2 hold rubber stoppers, through which thermometers are inserted. The animal is inserted through a small opening at C. Below A_1 and A_2 are tubes D_1 and D_2, half an inch in diameter, which can be inserted into dishes containing water or sulphuric acid of known specific gravity. The dishes containing sulphuric acid are covered to prevent alteration in specific gravity due to absorption of moisture.

The apparatus in this condition was used for experiments in which a humidity gradient was required. When constant humidity was needed, a further modification was introduced. A_3 was connected to a long glass-bead tower, which in turn was

nnected with two U tubes arranged in series, filled with glass beads. Sulphuric id of known concentration was introduced into the tower and the U tubes. A_1 was nnected to an air pump. A very slow current of air was then drawn through the hole apparatus. Tubes D_1 and D_2 were made air-tight by placing their ends into shes containing mercury.

All the experiments were carried out in an underground dark cellar at Birkbeck ollege, University of London. The annual range of temperature was between and 20° C. Experiments were carried out at between 14 and 18° C., the variation temperature at the time of any one experiment not exceeding 1° C. Diffuse light intensity of 2 Ferranti foot-candles was used throughout the experiments.

Woodlice were isolated and starved for 3 days before the beginning of each periment in dishes in which R.H. was maintained between 90 and 95%, and obably reached saturation under stones contained in them. To minimize the eber-Fechner effect, i.e. the fact that a response depends not on the actual uantity of stimulus, but on the proportion which the increase bears to the preceding imulus, the animals, when placed in the glass tube apparatus, were initially troduced into about 90% R.H., and left for 10 min. to recover from the effects handling. A gentle current of air bubbled through sulphuric acid of known ecific gravity was then drawn through the apparatus for 5 min. Preliminary periments had shown that such a slow rate of air flow did not influence the irection of movement of *P. scaber*. The apparatus was then made air-tight, and ne speed and the number of turnings performed by the animal were recorded very 30 sec. for a period of 1 hr. The number of 'rests', or periods of complete activity over a period of 30 sec., was also noted. Although the absolute speed ould be recorded by noting the number of inch divisions traversed per 30 sec., was impossible to determine the absolute number of turnings (i.e. klinokinesis, ccording to Gunn and the rate of change of direction, or r.c.d., according to Ullyott), as the shape of the apparatus did not allow for turning in all the possible irections. The animal moved about freely and could exhibit turnings of three ifferent types: (*a*) it could swing itself completely round and then proceed in the riginal direction, i.e. undergo an angular deviation of 360°; such movements in *P. scaber* were observed at low humidities only, and not once at relative humidities ver 85%; (*b*) the animal could swing around and proceed in the opposite direction, uch turning involving an angular deviation of 180°; (*c*) the animal could turn 90°, nd then swing back and proceed in the original direction. For the sake of convenience a turning of 90° was taken as a unit of measurement. Although the bsolute number of angular deviations could not be recorded, the relative number t different humidity conditions could be obtained, and for this reason they are of alue.

EXPERIMENTAL DATA

1) *Reactions of* Porcellio scaber *to air currents* (100 *and* 50% R.H., $t = 16$–$17°$ C.)

It was important to find out whether *P. scaber* orientated itself to air currents, ince these were used at the beginning of each 'constant humidity' experiment.

Six woodlice were introduced into a glass tube 12 in. long and ½ in. in diameter, and a gentle current of air was drawn through it by means of an air pump. The number of animals facing the current and of those turned away from it was recorded every 15 min. for a period of 2 hr. in the case of 100% R.H., and for a period of 1 hr. in the case of 50% R.H. The same animals were not kept for more than 1 hr. at 50% R.H., as they were in danger of being desiccated.

In both humidities forty readings of six animals in each case were recorded. A new set of six animals was used for each experiment. In the majority of cases woodlice were seen to aggregate at either end of the tube. The animals 'resting' or moving at right angles to the current were neglected.

At 100% R.H., $t = 16–17°$ C., 108 animals faced the current, 110 faced away from it. At 50% R.H., $t = 16–17°$ C., 96 animals faced the current and 101 faced away. From these results it was concluded that woodlice do not orientate themselves to slow air currents.

(2) *Thigmokinetic behaviour of* Porcellio scaber

It was observed that the thigmokinetic response characteristic of woodlice varied with relative humidity. A series of simple experiments was undertaken in this connexion. The animals were introduced into the 'alternative chamber' glass dish; constant humidity was maintained and altered between one set of experiments and the next by means of different concentrations of sulphuric acid. Temperature variation in the experiments ranged between 14 and 17° C., and within a single degree for any one experiment. Diffuse light of intensity of 2 Ferranti foot-candles was used.

Since the animals became progressively less active with the rise of relative humidity, the thigmokinetic response appeared to be closely associated with the hygrokinetic one. To distinguish between the two, the percentage of the time the woodlouse spent next to the wall of the glass dish while it was *still moving* was taken as the final indicator of the thigmokinetic behaviour. One woodlouse at a time was introduced and readings were taken every 15 sec. for a period of half an hour. To minimize the handling effect, readings were not taken in the first 10 min.

Twenty animals were taken through relative humidities of 20–25, 50–55, 70–75 and 90–95%. When the animals came to rest they invariably did so touching the wall of the vessel, with antennae folded ventrally and closely pressed against the solid surface. If the animals were placed in a rectangular dish, they usually came to rest in one of its corners. When the animal walked near the glass side, one of its antennae always touched the wall, even if the rest of the body was 1 mm. or so away from it. If one of the antennae be removed, the animal still keeps to the side, with the remaining antenna pressed against the glass surface. This seems to indicate that some of the organs of touch are localized in the antennae.

From Table 1 it can be assumed that the thigmokinetic response is dependent on the degree of humidity, and its intensity rises with the rise in relative humidity, increasing steeply on the approach to saturation.

Table 1. *Thigmokinesis of twenty* Porcellio scaber *in different relative humidities*

t = 14–16° C.	20–25 % R.H.	50–55 % R.H.	70–75 % R.H.	90–95 % R.H.
% of 30 min. spent in moving next to the wall (thigmokinesis)	81·6	79·5	85·9	92·6

(3) *Behaviour of* Porcellio scaber *under constant humidities*

Thirty *P. scaber* were kept at 90–95 % R.H. One at a time was introduced into e glass tube apparatus and allowed 15 min. before a gentle current of air was ssed through for 5 min. The air current was bubbled through sulphuric acid of nown specific gravity, and thus a known degree of relative humidity was obtained e Methods, p. 116). Three sets, of ten animals in each, were experimented on. ets I and III were taken through relative humidities of 0–10, 20–25, 40–45, 58–63, 0–85, 90–95 and 98–100 %. Set II was taken through relative humidities of 0–10, 0–45, 58–63, 68–73, 85–90 and 98–100 %. Readings were taken every 30 sec. by stop-clock, and four factors were noted in the behaviour of each animal:

(*a*) *activity*, as shown by the actual number of inches passed through by an nimal in a given period of time;

(*b*) *speed*, which differs from activity in that it shows the actual velocity while ie animal is moving, disregarding the periods of rest;

(*c*) *the number of turnings* (klinokinesis) in a given time;

(*d*) *the number of rests*, i.e. periods of complete inactivity for 30 sec. Clearly r) and (*d*) are two different methods of expressing the same facts.

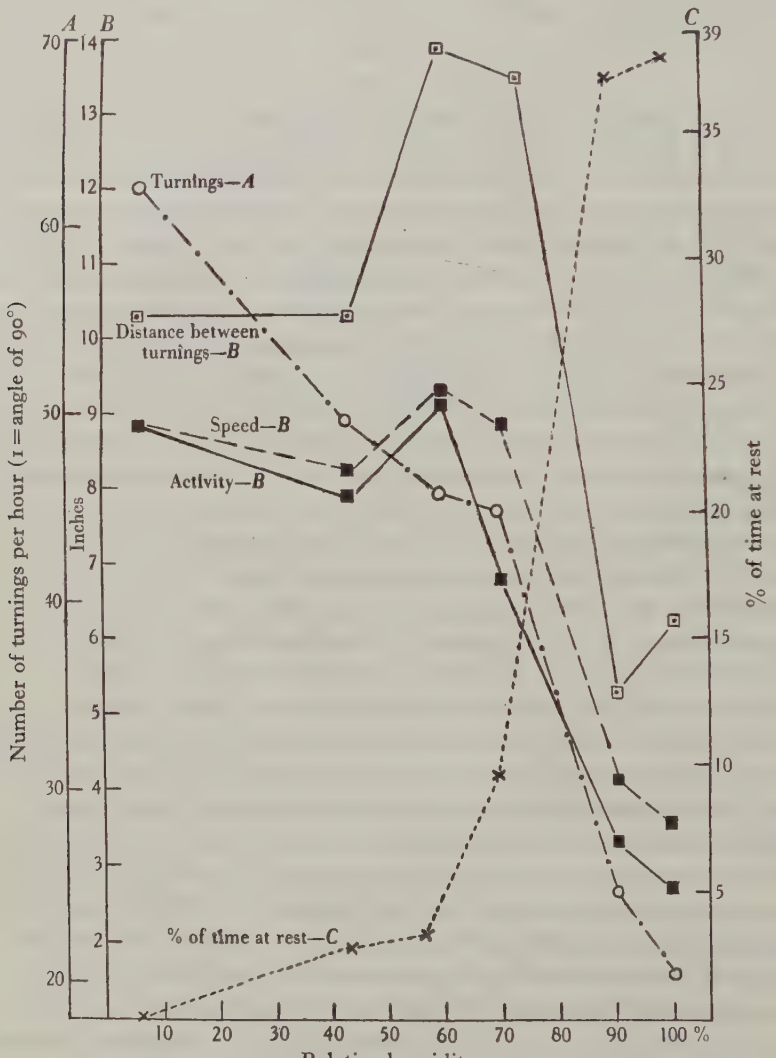
The results obtained from these experiments are shown in Graphs 1 and 2.

As can be seen in Graph 1 the number of turns, speed and activity decrease with ie rise of relative humidity, while correspondingly there is a rise in the number f 'rests'. Whereas the fall of the number of turns with the rise of relative humidity continuous, the maximum speed and activity were obtained at 58–63 % R.H. It is nteresting to note that Gunn (1937) has observed that the intensity of reaction was greatest between 35 and 65 % R.H. approximately, and that at 55 % R.H. there as a definite reaction to a difference of 6 % R.H.' He further notes that the intensity f the reaction diminishes in the higher and lower humidities.

Speed and activity are almost identical up to 68 % R.H., being greatest at 58–63 % .H. (average speed 9·38 in., average activity 9·34 in. per min. for a period of 1 hr., hen sets I, II and III are taken into account. When the averages of twenty animals ets I and III) are taken into account, the greatest speed and activity are obtained t 20–25 % R.H. (speed 9·98 in., activity 9·88 in. per min.).

Appreciable difference between speed and activity was first observed at 68–73 % .H. (speed = 8·88 in., activity = 6·82 in. per min. from sets I, II and III). This howed that although speed was maintained at almost the same level as at lower elative humidities, activity was already decreasing. Speed is, therefore, not an ndicator of hygrokinesis, but is only one of its factors. Above 68–73 % R.H. both

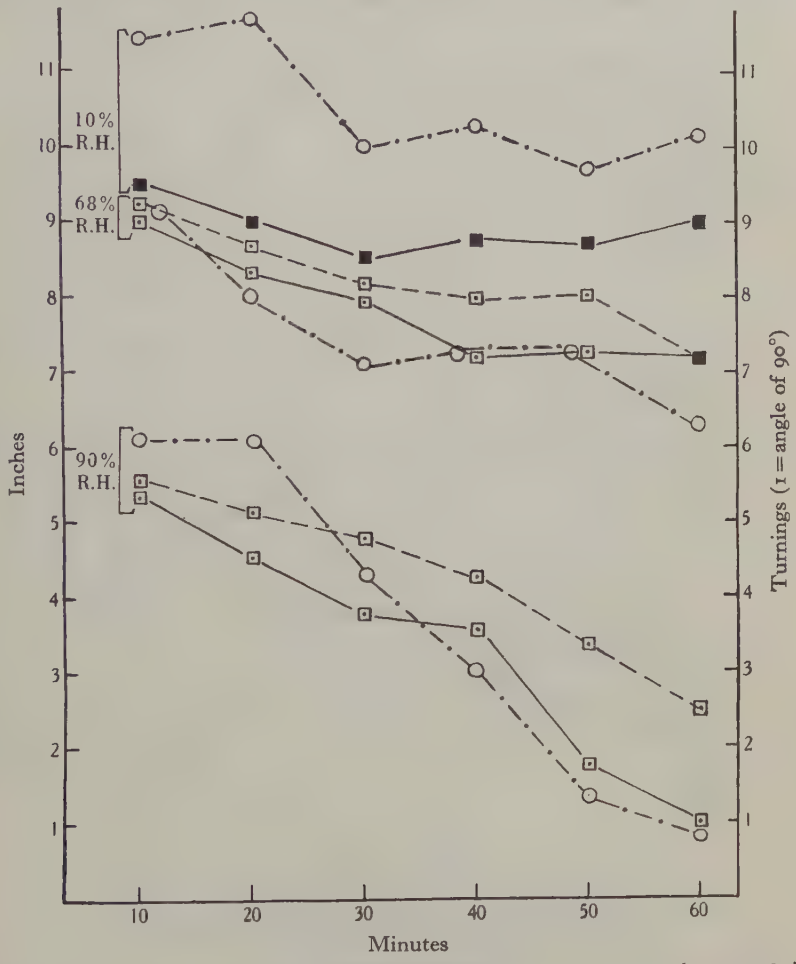
speed and activity fell off with the rise of humidities. However, as stated above, there was a steady fall in the number of turnings (1 turning = 90°) with the rise of relative humidity. Whereas at 0–10% R.H. the average number of turnings was 61·95 per hour, at 98–100% R.H. it was 20·33.



Graph 1. Behaviour of the woodlouse *Porcellio scaber* in constant humidities, calculated from the average of 30 specimens. The number of turnings is read from the ordinate *A*. The average distance between turnings, the activity and the speed per minute from the ordinate *B*. The percentage o time at 'rest' is read from ordinate *C*.

Attempts were made to correlate the variables by dividing the total distance travelled by the animal in 1 hr. by the number of turnings per hour, i.e. to estimate the average distance between turnings. The figures obtained are those shown in Table 2 and Graph 1. It is thus seen that not only the total number of turns bu

distance between turns decreases with the rise of humidity. This apparent crepancy is due to a large number of 'rests' and the low activity of the animals high humidities. The smaller distance between turnings, together with decreased ivity will tend to restrict the woodlice to the regions of higher or 'optimum' midity. The significance of this result is discussed later (see p. 134).



■——■ Activity and speed coinciding. ⊡——⊡ Activity. ⊡- - -⊡ Speed. ○---○ Turnings.

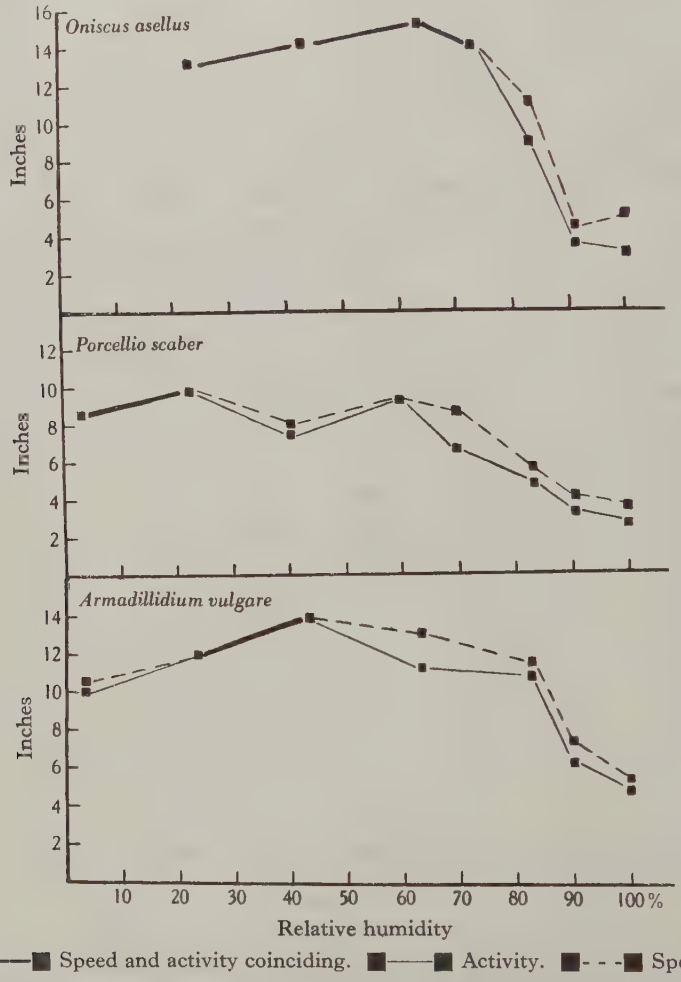
raph 2. The behaviour of *Porcellio scaber* (average of 30 specimens) throughout the 1 hr. experients at 10% R.H., 68% R.H. and 90% R.H. The average activity and speed per minute, and the amber of turnings are recorded for every 10 min.

Table 2. *Distance travelled between turnings obtained from the averages of thirty* Porcellio scaber

0–10% R.H.	40–45% R.H.	58–63% R.H.	68–73% R.H.	90–95% R.H.	98–100% R.H.
10·3 in.	10·4 in.	13·9 in.	13·5 in.	5·3 in.	6·2 in.

(4) *Comparison of behaviour of* Oniscus asellus *Lin.*, Porcellio scaber *Latr. and* Armadillidium vulgare *Latr. under similar constant humidities*

The methods used in observations on *Oniscus asellus* and *Armadillidium vulgare* were identical to those employed in constant humidity experiments on *Porcellio scaber*. It was of interest to see whether there were any differences in behaviour

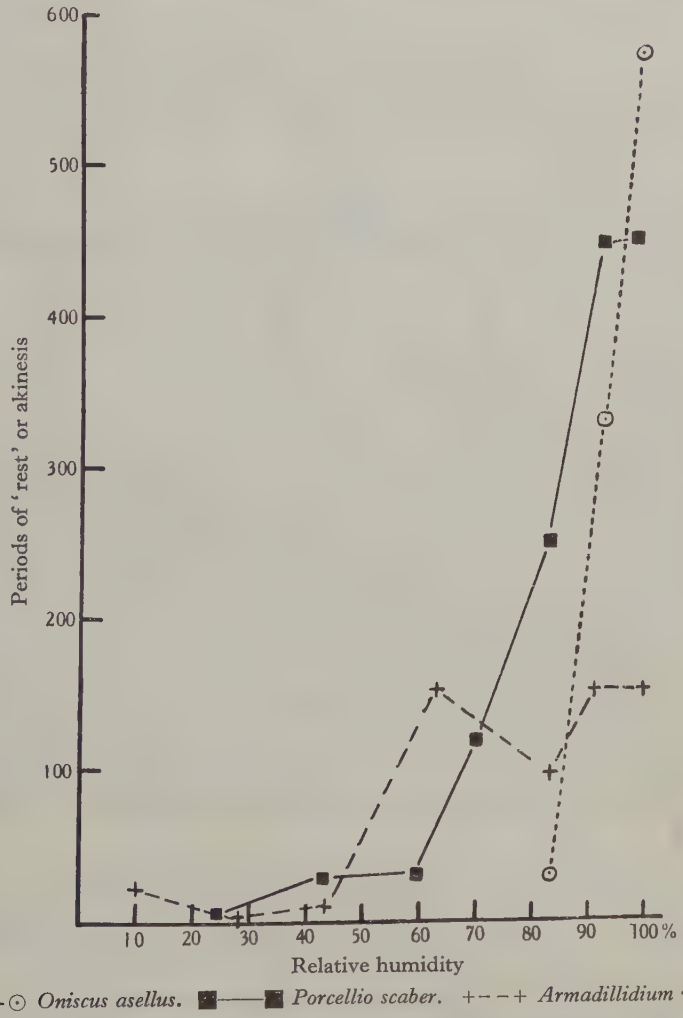


Graph 3. Average activity and speed per minute of *Oniscus*, *Porcellio* and *Armadillidium* in relation to constant humidities.

which could be correlated with the differences in their natural habitats. Both *P. scaber* and *Oniscus asellus* are restricted to damp situations under stones, humus bark of decaying logs and pieces of wood, but although the habitats of the two species overlap to a great extent, *Porcellio scaber* is never found in contact with actually wet undersurfaces of stones or wood, whereas *Oniscus asellus* frequently is

nadillidium vulgare, which may be considered to be one of terrestrial isopods t adapted to life on land, is restricted to drier calcareous soils.

Ten specimens of *Oniscus* were observed in relative humidities of 20–25, 40–45, 65, 70–75, 80–85, 90–95, 98–100 %, and ten specimens of *Armadillidium vulgare*



Graph 4. The number of periods of 'rest' (30 sec. of complete inactivity) in relation to constant humidities, in *Oniscus*, *Porcellio* and *Armadillidium*.

relative humidities of 0–10, 25–30, 40–45, 60–65, 80–85, 90–95 and 98–100 %, emperatures between 14 and 18° C. The animals chosen for all experiments were nearly as possible of the same size, and varied between 1·25 and 1·65 cm.

In all essentials the behaviour of the three species was the same, namely, with rease of relative humidity there was a decrease in activity and speed, i.e. hygro- esis was clear in all cases (Graph 3), e.g.

% R.H.	Speed per minute (in.)		
	Oniscus asellus	*Porcellio scaber*	*Armadillidium vulgare*
20–25	13·24	9·98	12·04
60–65	15·30	9·38	13·04
90–95	4·5	4·16	7·6

The number of 'rests', i.e. 30 sec. of complete inactivity, increased consistentl with the rise of relative humidity in the cases of *Oniscus* and *Porcellio*, but remaine at approximately the same level from 60 to 100% R.H. in *Armadillidium* (Graph 4) e.g.

'Rests'

% R.H.	10 *Oniscus asellus*	10 *Porcellio scaber*	10 *Armadillidium vulgare*
25	0	4	0
60–65	2	40	155
80–85	38	258	97
90–95	343	448	157
98–100	574	457	159

The smaller and more constant number of 'rests' of *A. vulgare* may be a expression of its lesser sensitivity to humidity. This supposition is borne out b the subsequent experiments which show the greater resistance of this species t desiccation (see p. 128). In this connexion it is interesting to note that althoug *A. vulgare* can roll itself into a sphere, at no time was 'rolling up' observed at lo humidities; on the contrary, when the isopod was left in the apparatus for severa hours it remained active until it became moribund on desiccation.

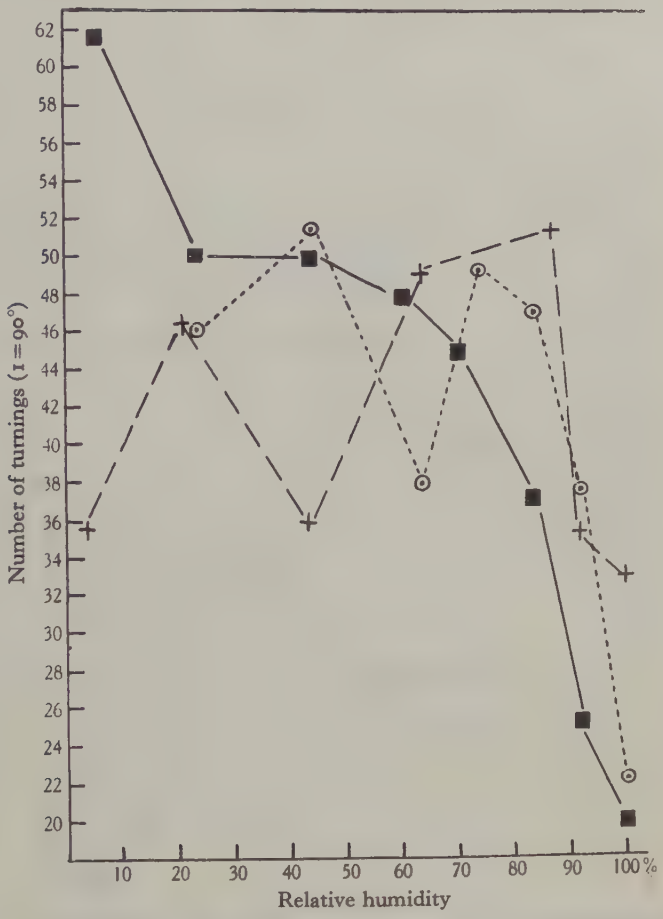
The total number of turnings per hour of *Porcellio* and *Oniscus* decreased wit the rise of relative humidity, but *Armadillidium* did not show any appreciabl corresponding decrease (Graph 5). However, the distance travelled betwee turnings, obtained by the ratio of total distance/number of turns decreased ver rapidly with the rise in humidity in *Oniscus*, and to a lesser extent in *Armadillidium* e.g.

% R.H.	Average distance between turns (in.)		
	Oniscus asellus	*Porcellio scaber*	*Armadillidium vulgare*
25	26·5	10·9	16·7
45	21·1	10·4	22·3
60–65	25·9	13·9	14·5
90–95	7·9	5·3	10·8
98–100	6·6	6·2	9·4

See also Graph 6.

It thus seems that the mechanism by which isopods are retained in areas higher humidities, namely, hygrokinesis and increase in the frequency of turn are operative in all the three species, but are best developed in *Oniscus asellus*. Th indicates the greatest sensitivity of this species to humidity, and is probab associated with its most rapid loss of water by evaporation (see experiments

esiccation, p. 128). The lesser sensitivity to humidity of *Armadillidium vulgare*, obably correlated with its greater resistance to desiccation, was indicated by the aller number of periods of inactivity or 'rests'.



⊙- - -⊙ *Oniscus asellus.* ■——■ *Porcellio scaber.* +– –+ *Armadillidium vulgare.*

Graph 5. The average number of turnings (angle of 90° taken as unit) per hour in *Oniscus*, *Porcellio* and *Armadillidium*, in relation to constant humidities.

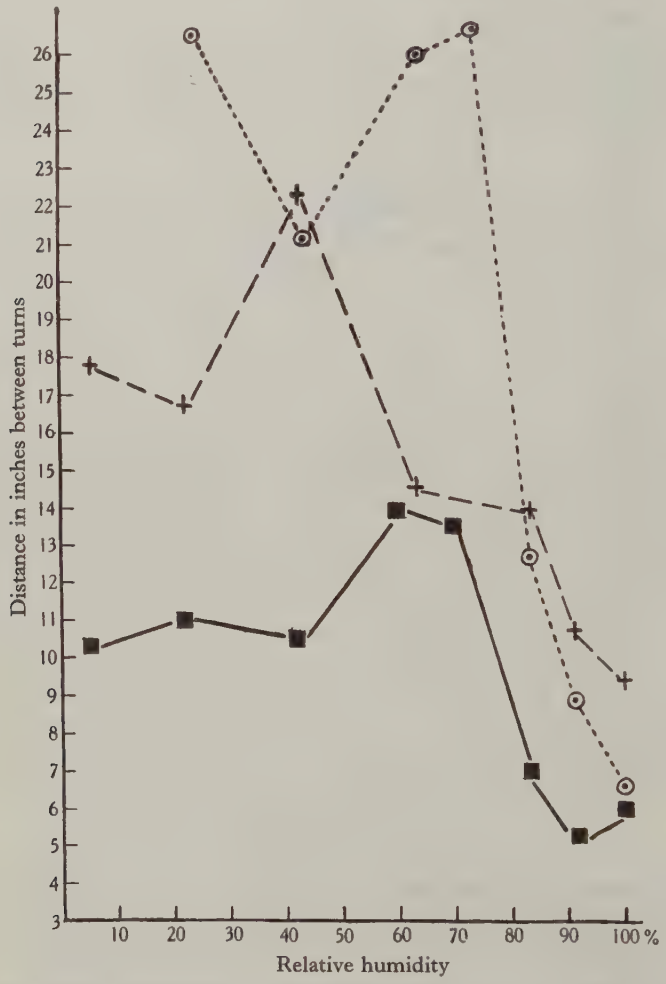
It was interesting to note that the specimens of *Oniscus asellus* and *Armadillidium ulgare* were consistently more active than those of *Porcellio scaber*. Moreover, uch less individual variation was seen in the first two species than in the last. bbott (1918) in experiments on photic responses also noted that the behaviour f *Oniscus* was much more stereotyped than that of *Porcellio*.

(5) *Behaviour of* Porcellio scaber *in humidity gradients*

The method of obtaining humidity gradients has already been described (see Methods, p. 116).

Altogether fifty-five *Porcellio scaber* were experimented with:

10 in 75–95 % R.H. ($t = 15$–$18°$ C.),
10 in 65–85 % R.H. ($t = 14$–$15°$ C.),
10 in 55–75 % R.H. ($t = 14$–$15°$ C.),
18 in 20–73 % R.H. ($t = 14$–$18°$ C.),
7 in 42–60 % R.H. ($t = 14$–$15°$ C.).



⊙- - -⊙ *Oniscus asellus.* ■——■ *Porcellio scaber.* +– –+ *Armadillidium vulgare.*

Graph 6. The average distance between turnings in relation to constant humidities, in *Oniscus*, *Porcellio* and *Armadillidium*.

In each case one animal at a time was introduced into the glass tube apparatus fo a period of 1 hr. Readings were not taken for the first 10 min. to allow for recover from handling. The results of the experiments are given in Table 3. The numbe of turns, speed and the number of 'rests' were recorded every 30 sec., and th behaviour of the animal in the damp half of the apparatus was compared with tha in the dry half. In each case the following points were calculated:

(1) Percentage of total time spent in damp half of the apparatus.
Percentage of total time spent in dry half of the apparatus.
(2) Percentage of total time spent at 'rest' in the damp half.
Percentage of total time spent at 'rest' in the dry half.
(3) Average speed per minute in damp half.
Average speed per minute in dry half.
(4) Total number of turns in damp half.
Total number of turns in dry half.
(5) Average distance travelled between turns in the damp half.
Average distance travelled between turns in the dry half.
(6) Average time interval between turns in the damp half.
Average time interval between turns in the dry half.

Table 3. *Behaviour of* Porcellio scaber *in humidity gradients*

	% of total time spent in		% of total time spent at rest in		Average speed (in.) per minute in		Total no. of turns (1 = 90°) in		Average distance (in.) between turnings in		Average no. of minutes between turnings in	
	Damp half	Dry half	Damp half	Dry half	Damp half	Dry half	Damp half	Dry half	Damp half	Dry half	Damp half	Dry half
5–95 % R.H. = 15–18° C. 10 *P. scaber*	87·17	12·83	50·42	0	4·48	7·48	143	41	5·99	13·65	1·32	1·61
5–85 % R.H. = 14–15° C. 10 *P. scaber*	73·75	26·25	34·67	0	5·52	8·00	183	106	8·55	18·89	1·42	2·21
5–75 % R.H. = 14–15° C. 10 *P. scaber*	70·83	29·17	26·03	6·25	5·70	6·56	186	80	6·59	11·89	1·38	2·21
0–73 % R.H. = 14–18° C. 18 *P. scaber*	74·49	25·51	29·03	0·18	5·38	6·52	347	255	7·73	6·05	1·39	0·99
2–60 % R.H. = 14–15° C. 7 *P. scaber*	67·71	32·29	23·57	0	6·68	7·60	113	164	10·54	5·46	1·51	0·73

Examination of Table 3 shows that in all the humidity gradients provided, the imals spent the greater part of the hour in the damper half of the apparatus. eed was consistently higher in the dry half and the number of 'rests' greater in e damp half.

In the gradients of 75–95, 65–85 and 55–75 % R.H. the average distance between rnings and the time interval between turnings are shorter in the damper half of e apparatus. Such behaviour, associated with hygrokinesis (indicated by the ger number of 'rests' and lower speed in higher humidities), would tend to restrict e isopods to these conditions. These results are consistent with those obtained experiments with constant humidities. No such correlation, however, was tained when the animals were subjected to gradients of 20–73 and 42–60 % R.H., r although speed was again higher in the dry half, the greater part of time spent the damp half and the periods of 'rest' almost entirely restricted to the damp

half, the average distance, and the average time interval, between turnings, were greater in the drier part of the apparatus, and such results are inconsistent with any which were previously obtained. No satisfactory explanation for this difference in behaviour can be given, but it may be suggested that the normal behaviour pattern was altered by the continual physiological instability caused by loss of water by evaporation at low humidities. It is also probable that behaviour alters with the steepness of gradient, for whereas in four sets of experiments the gradient was approximately that of 20%, it was 53% between 20 and 73% R.H.

(6) *Loss of water by evaporation*

It was interesting to see how far the differences in behaviour of woodlice in different humidities could be associated with the rate of loss of water by evaporation, and the following series of simple experiments were undertaken in this connexion.

(*a*) *Duration of life of* Oniscus asellus, Porcellio scaber *and* Armadillidium vulgare *under different relative humidities.*

Twenty specimens of each species, of approximately the same size (1·25–1·65 cm.) previously starved in 100% R.H. for 3 days, were placed in desiccators at 0, 25, 50, 75, 85, 93 and 100% R.H. ($t = 15–18°$ C.). To prevent conservation of water due to possible formation of groups, each isopod was placed in a separate desiccator and the number of hours of its survival was noted (see Table 4). It was

Table 4. *Duration of life of* Oniscus asellus, Porcellio scaber *and* Armadillidium vulgare *at different relative humidities* ($t = 14–18°$ C.). *Number of hours of survival based on the averages of twenty animals in each case*

R.H. %	*Oniscus asellus*		*Porcellio scaber*		*Armadillidium vulgare*	
	Average	Limits	Average	Limits	Average	Limits
0	4·3	3–5	5·27	4–6	7·15	6–10
25	6·85	5–7·5	8·17	6·5–10	9·92	6–16
50	6·2	5–8	10·17	6–16	30·15	16–44
75	16·25	12–20	25·25	18–44	59·15	36–87
85	25·5	11–28	29·0	18–43	65·65	35–96
93	33·17	16·5–75	39·17	20–67	114·6	49–240
100	—	32 hr. to over a month	—	47 hr. to over a month	—	76 hr. to over a month

found that the resistance to desiccation determined by death-point was consistently greatest in *Armadillidium vulgare* and greater in *Porcellio scaber* than in *Oniscus asellus*. The figures in Table 4 are probably purely relative, and do not represent the exact survival under natural conditions, since in the desiccators the whole ventral side of the animal, as well as the dorsal, is exposed to uniform humidity. Normally the ventral surface of the animals at rest is closely pressed against a solid surface and undoubtedly a microclimate of higher humidity is thus retained, at least for some time.

Estimation of water loss by evaporation of Oniscus asellus *during the* 1 *hr. experiments.*

(i) As before, twenty specimens of *Oniscus asellus*, starved for 3 days at 100% ., were weighed and placed in desiccators at 0, 50 and 75% R.H. for a period of r., after which the animals were reweighed. The following figures, taken from average of twenty animals, represent the percentage loss of water by evaporation hour:

0% R.H., $t = 16$–$18°$ C.	50% R.H., $t = 15$–$18°$ C.	75% R.H., $t = 18°$ C.
9·9%	6·3%	3·6%

er each experiment the animals were returned to 100% R.H., left for 24 hr. and eighed. With the exception of four cases out of sixty, the animals gained in ight. This gain varied widely and could not be correlated either with the original ight of the animal or its previous percentage loss by evaporation.

(ii) As a check to the above experiments another set was undertaken. Ten or nty specimens of *Oniscus* were placed in 95, 75, 50 and 20% R.H. for 1 hr., woodlouse to a desiccator. Each animal was then weighed and placed in a ighed specimen tube containing anhydrous copper sulphate, from which it was arated by a strip of perforated zinc. The tubes were placed in a drying oven for hr.—1 hr. at 100° C. to stop the action of enzymes, and 23 hr. at 60° C. After hr. the animals and the specimen tubes were reweighed. The water content of *asellus* after 1 hr. at different relative humidities ($t = 15$–$16°$ C.) was calculated l is shown in the following figures:

Relative humidity %	95	75	50	20
Water content %	63	59·6	57·3	57·2
No. of specimens	20	10	20	10

nce the loss of weight is mainly due to evaporation of water on drying, it can be ncluded that at the end of 1 hr. the animals kept at 95% R.H. have a much higher ter content than those kept at lower humidities; moreover, the evaporation of ter in *Oniscus* exposed to dry conditions appears to be rapid. It must be remem- red that *O. asellus* may be regarded as an isopod little adapted to a terrestrial bit, and is found in habitats where humidity must be nearly always at saturation int.

(iii) The rate of loss of water in *O. asellus* during one hour was then calculated 0 and 50% R.H. ($t = 14$–$17°$ C.). One animal at a time was placed in a small x of perforated zinc attached to the arm of a balance by a fine wire. The zinc x was suspended in a small vessel at 0 and 50% R.H. This method gave a set continuous readings, but its chief disadvantage was the disturbance of the tial humidity on the introduction of the zinc box. The rate of loss of weight is own in Table 5.

Table 5. *Ten* Oniscus asellus *weighed for* 1 *hr. at* 0 *and* 50% R.H.

Minutes	5	10	15	20	25	30	40	50	60
% loss of weight at 0% R.H.	0·4	1·1	1·6	2·3	2·8	3·6	4·4	5·7	6·4
% loss of weight at 50% R.H.	0·3	0·7	1·3	1·6	2·0	2·4	3·0	3·9	5·6

From all the above results it may be concluded that the loss of water by evaporation in *O. asellus* is proportional to time and to the percentage of relative humidity. It may be suggested that the differences in the activity of woodlice are due to the continuous alteration in their water content, the loss of which does not appear to be regulated.

(7) *The effect of loss of water by evaporation on phototaxis of* Oniscus asellus

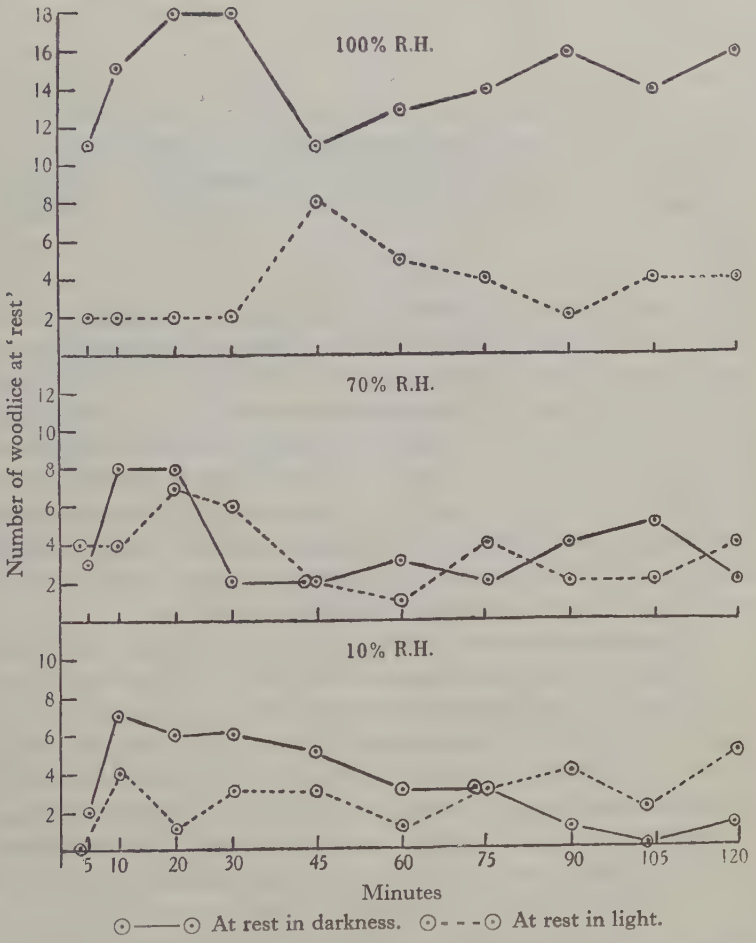
The present series of experiments are here included, as they indicate that there is a balance between phototaxis and humidity reactions of *Oniscus asellus*.

Abbott (1918) in phototactic experiments on *Porcellio scaber* and *Oniscus asellus* found that the two species are sensitive to a range of light from 100 to 0·01 c.m. the response being the same for all light intensities. He also states that the reaction of *Oniscus* was essentially the same whether the animal had been previously kept in maximum or minimum moisture, but notes that *Porcellio* was less negative after living in a dry habitat. The same tendency to reversal of phototactic reaction in *Porcellio*, and a definite reversal from negative to positive phototaxis after a period of desiccation in *Armadillidium* has been described by Henke (1930).

In the present set of preliminary experiments on phototaxis of twenty specimens of *Oniscus asellus* under constant humidity conditions of 100, 93, 75, 70, 60, 50, 40, 25 and 10% R.H. at temperature 14–15° C. and at light intensity of 45 Ferranti foot-candles, it was noted that the animals show definite negative phototaxis above 75% R.H. Below 75% R.H. the initial negative phototaxis seems to disappear, and the animals appear indiscriminately on the illuminated and shaded halves of the apparatus. Below 40% R.H. there is an indication that a reversal from negative to positive phototaxis occurs, as the number of the woodlice at rest in the light half of the apparatus is equal or greater than in the dark half (see Graph 7). These experiments, although in their initial stages, suggest that a reversal from negative to positive phototaxis, probably associated with water loss by evaporation, occurs in *O. asellus*.

The effect of humidity gradients on the phototaxis of *O. asellus* was next observed. It is known that when land isopods are introduced into an 'alternative chamber', with low humidity at one end and high humidity at the other, they aggregate in the damp half of the apparatus (Gunn, 1937). Preliminary and control experiments have also shown that in saturated air *O. asellus* invariably comes to rest in the shaded half of the apparatus. However, when the animals are placed in the alternative chamber with the range of 50–90% R.H. ($t = 18°$ C.), the drier half being screened with black paper and the damper half exposed to diffuse light of intensity of 45 Ferranti foot-candles, the isopods at first wander about the apparatus indiscriminately. The behaviour of forty specimens of *Oniscus*, previously kept in darkness at 100% R.H. for 3 days, was noted under such conditions. One animal was introduced into the apparatus at a time to prevent the possible conservation of water due to the formation of aggregations (Allee, 1926). Each animal was weighed before and after each experiment.

Six out of the forty animals came to rest in the dark, dry half of the apparatus nd eventually died of desiccation; five settled in the dark for a short period of me and then continued to move until they finally came to rest in the light damp alf;



raph 7. Tendency to reversal from positive to negative phototaxis, at low humidities, in *Oniscus rellus* (20 specimens). The numbers of animals at rest in the shaded and illuminated (diffuse light f 45 Ferranti foot-candles) halves of the apparatus at 10, 70 and 100 % R.H. are recorded.

Eight specimens moved continuously in both halves of the apparatus, settling nally in the light damp half;

Twenty-one others, after much initial activity, came to rest in the dark dry half, ut then moved to the light damp half and came to rest there. 15 min. at rest in ne light half was taken to indicate the final position of each animal.

The initial and final weights of the woodlice, the percentage loss of weight by vaporation, and the time taken to come to rest in the light damp half were recorded. The average loss of weight was 6·2 % of the original body weight (limits 2·5 and

10·2%). No correlation was observed between the weight of the woodlice and the percentage loss of water. The time taken to come to rest in the light varied between 20 min. and 3 hr. 20 min.:

Twenty-six out of thirty-four settled in the light half in between 50 min. and 2 hr.;

Four in under 50 min.; and

Four in over 2 hr.

It thus seems that the initial negative phototaxis is stronger than the humidity reaction of *Oniscus*. However, the increased activity of woodlice, due to the loss of water by evaporation and the masking or the reversal of the negative photic reaction, combine to retain the animals in the regions of greater humidity.

DISCUSSION

The three species of isopods studied, namely, *Oniscus asellus* Lin., *Porcellio scaber* Latr. and *Armadillidium vulgare* Latr., are widely distributed and common in Europe and North America. The animals are restricted to damp situations in which the microclimatic humidity conditions approach those of saturation. They are members of a very old stock in which there is but little morphological plasticity. Their stereotyped behaviour, controlled to a great extent by external stimuli, may be associated with the primitive scalariform, vermian type of nervous system. The brain of *Porcellio*, characterized by only slightly developed sight centres, and absence of antennal glomeruli and corpora pedunculata, is considered to be one of the most primitive of Arthropod brains (Hanström, 1928).

One of the most important adaptations of isopods to land, which, however, is not found in all species, is the development of tracheae on the pleopods, and hence of aerial respiration. The endopodites of woodlice are damp with a distinct film of water, and retain their 'gill-like' character. These are the only respiratory organs in *Oniscus*, which is capable of picking up drops of water by the movements of its telson and uropods, and conveying them to the respiratory surfaces through minute channels (Verhoeff, 1920). In *Porcellio* and *Armadillidium* while the exopodites are invaginated to form small branching air tubes, the endopodites are 'gill-like', and the isopods seek conditions for both methods of respiration.

Miller (1938) has found that the optimum relative humidity for terrestrial isopods is close to 100%, and that there is an indication that survival is inversely proportional to the saturation deficiency. In the present set of experiments it was seen that the resistance to desiccation was consistently greatest in *Armadillidium*, and greater in *Porcellio* than in *Oniscus*. However, all the three species survived for indefinitely long periods only in desiccators at 100% R.H. From experiments on *O. asellus* it could be concluded that the peril of desiccation is a very real one to woodlice, for not only the respiratory organs are affected, but there appears to be no control of loss of water through the cuticle, the loss of weight due to evaporation being proportional both to relative humidity and the time. It was noted that although *Armadillidium vulgare* is able to roll up into a ball, at no stage in the desiccation experiments was it seen to do so. It may be that rolling up is a response

rapidly changing conditions, rather than to a continuous stimulus of constant w relative humidity.

Allee (1926) has suggested that the formation of aggregations by woodlice is of rvival value, since the loss of water of a group is much lower than that of an lated animal. The difficulty of keeping isolated woodlice in laboratory conditions is continually noted. No doubt the animals not only live in a microclimate, but oduce a microclimate of their own, for while the dorsal surface of the body is posed, the damp pleopods of the ventral surface are normally closely pressed ainst the substratum, and under such circumstances the air in contact with the ver surface must be at saturation point. The isopods which survive for any length time at 90% R.H. in laboratory conditions, provided they are in contact with a lid surface, die of desiccation when the whole body is exposed to this humidity.

The survival of isopods on land has undoubtedly been favoured by the develop- ent and combination of hygrokinetic, thigmokinetic and negatively phototactic haviour. It was seen that the degree of thigmokinetic response of *Porcellio scaber* es with the relative humidity of the air. The correlation which exists between grokinesis and thigmokinesis is significant and can also be considered of survival lue, since it prevents the adherence of animals to solid surfaces in dry conditions, d hence their desiccation.

The effect of water loss on behaviour of woodlice was clearly shown by *Oniscus ellus*. According to modern terminology (Fraenkel & Gunn, 1940) this species ows low hygrokinesis and is negatively phototactic. When these woodlice were troduced into a humidity gradient, the drier end of which was shaded, and the mper exposed to diffuse light, the initial negative phototaxis was stronger than e reaction to humidity, but with the loss of water by evaporation, there was a asking or reversal of the reaction, which together with increased activity, brought e animals to the regions of 'optimum' humidity. Such an alteration in the actions is of general interest, since it clearly indicates that the behaviour of an ganism is not fixed, but consists of variables depending on the balance between e external and internal environments.

From the foregoing it is clear that the humidity reactions are of primary portance to terrestrial isopods. The behaviour of *Oniscus asellus*, *Armadillidium ulgare* and *Porcellio scaber* in constant humidities, and of the last-named species humidity gradients, was observed, and it appears that there are two mechanisms hereby the animals are retained in the damper zones:

(1) *Hygrokinesis*, or the decrease of activity with the rise of relative humidity, as seen in all the three species. Gunn (1937) has demonstrated hygrokinesis in *orcellio scaber*, but further details of this mechanism were obtained, as it was ound that it is expressed not only by the number of minutes during which the nimals are moving, or are at complete rest at any constant humidity, but also by he variations in the speed of the movements in different humidities. At high umidities activity and speed are consistently low, and the animals tend to become ompletely akinetic. This is true of *Oniscus* and *Porcellio*, in the case of *Armadillidium*, n the other hand, although the speed again decreases with the rise of humidity,

the number of 'rests', i.e. of periods of akinesis, remains relatively low and constant above 65 % R.H. In conditions below 85 % R.H. ($t = 14$–$18°$ C.) all the three species remained active to the end of the 1 hr. experiments.

Hygrokinesis clearly results in bringing the animals to the regions of 'optimal', i.e. high humidity. Such a response is considered to be a primitive one (Mast, 1938), and is widely distributed throughout the animal kingdom.

(2) In addition, there appears to be a more complicated mechanism whereby the animals are retained in the 'optimal' zone, for when the total number of turnings is divided into the distance passed by the animals in the 1 hr. experiments, it becomes clear that the distance between turnings decreases with the rise of humidity and increases with its fall. This change in the behaviour pattern may be associated with the loss of water by evaporation from the animals, and together with the hygrokinetic effect, which in *Oniscus* and *Porcellio* results in complete inactivity at 90–100 % R.H., is effective in retaining the isopods in moist air. The decrease of distance between turnings with the rise of humidity has been observed in all the three species. In humidity gradient experiments on *P. scaber* this mechanism was again exhibited in gradients of high, but not in those of low, humidity.

It is interesting to note that Gunn & Pielou (1940) describe a similar mechanism designated as 'virtual inactivity' in the humidity reactions of *Tenebrio molitor*, which in contrast to the isopods aggregate at the drier end of a humidity gradient.

Although the mechanisms of these reactions in *Oniscus*, *Porcellio* and *Armadillidium* are the same, some differences in their intensity have been detected. The greater activity, at high humidities, of *Armadillidium*, as compared to that of *Porcellio* and *Oniscus*, may be an expression of its lesser sensitiveness to alterations in humidity and of its greater resistance to desiccation. The mechanisms are most clearly expressed in *O. asellus*, which, of the three species, must be considered the least adapted to land.

When a comparison is made of the mechanisms of the reactions of isopods with photophobotaxis described by Ullyott (1936) in the planarian *Dendrocoelum lacteum*, two main differences (among others) emerge, viz.:

(1) No kinetic effect was shown in *D. lacteum*, whereas hygrokinesis is clear in the isopods.

(2) Sensory adaptation is the basic factor determining the photic responses of *D. lacteum*. The meaning of sensory adaptation is that the effect of a continuous stimulus is not uniform, but that the intensity of the reaction produced by it decreases with the time and gradually dies down (Hecht, 1919). No such adaptation was satisfactorily demonstrated in the isopods, and it was particularly difficult to approach this question, since at low humidities woodlice are continually losing water by evaporation, and hence not maintaining a balanced physiological state.

As to the humidity receptors, none are known in woodlice (Gunn, 1937). It can be suggested that the rapid loss of water by evaporation and the consequent concentration of body fluids would in itself have an effect on the reactions of the animals. Any sense organs connected with this mechanism would be proprioceptive. If this suggestion is correct and there are no specialized receptors, no sensory adaptation need take place, and this is borne out by experimental results. This,

owever, would not account for immediate reactions. Gunn (1937) has suggested at the receptors probably lie in the thoracic region. It has been continually noted at the relatively delicate thoracic appendages are the first to show the effects of siccation, and it may be that they function as the 'hygrometers' of the body.

In conclusion, it may be said that the terrestrial isopods are the only group of rustacea which are able to live on land throughout the whole of their life cycle. owever, they are just able to survive the land conditions, not so much by morpho- gical adaptations, as by evolving a series of patterns of behaviour, which restrict em to moist dark habitats. In other words, they are able to survive on land by oiding the typical land conditions.

SUMMARY

1. The humidity reactions of *Oniscus asellus*, *Porcellio scaber* and *Armadillidium ulgare* have been analysed and compared.

2. The mechanism whereby the three species collect in moist air is twofold, nsisting of (*a*) hygrokinesis, or decrease in activity and speed in moist air, and) of more frequent turnings in space, retaining them in the areas of greater midity.

3. These mechanisms are most clearly expressed in *Oniscus asellus* and least in *rmadillidium vulgare*. This sequence may be correlated with the resistance to siccation of the three species, which is greatest in *Armadillidium vulgare*, and eater in *Porcellio scaber* than in *Oniscus asellus*.

4. It is suggested that the humidity reactions of isopods are controlled by water ss by evaporation from the whole body.

5. A correlation between hygrokinesis and thigmokinesis was observed in *orcellio scaber*.

6. There appears to be a reversal from negative to positive phototaxis in *niscus asellus*, correlated with the water loss by evaporation.

7. The humidity reactions, low thigmokinesis and negative phototaxis combine o retain the isopods in damp, dark habitats.

I wish to express my sincere gratitude to Prof. H. G. Jackson for continual help nd encouragement in the carrying out of this work, and my indebtedness to Mr Alastair Graham and Dr D. L. Gunn for helpful criticism and advice.

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THE PIGMENTATION OF CAVERNICOLOUS ANIMALS

I. THE PIGMENTS OF SOME ISOPOD CRUSTACEA

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INTRODUCTION

The colonization of caves, and the evolution of the peculiar characteristics of most members of the cave fauna, have interested many workers. The cave environment is of a specialized nature, its most obvious characteristics being absence of light, lack of food, uniformly low temperature and, in subterranean waters, low oxygen content. Cave animals are likewise specialized and commonly exhibit loss of pigmentation, development of specialized taste and tactile organs, together with degeneration of the eyes (*vide* the accounts in Spandl (1926) and in Chappuis (1927)). We felt that a comparison of the pigments of cave animals and of nearly related above-ground epigean species might throw some light on the reduction of pigmentation so commonly met with in cave animals. We decided to examine some isopod and amphipod Crustacea in particular, since these families have many subterranean and closely related epigean species and genera, and the literature includes experimental work on the inheritance of the reduced pigmentation in a typical subterranean (hypogean) isopod, *Asellus aquaticus covernicolus* Rac., together with observations on the development of pigmentation in cavernicolous Amphipoda when they are exposed to light, and on the loss of pigmentation in epigean Amphipoda kept in darkness.

Several lines of investigation suggested themselves. In the first place it seemed desirable to study the action of light on the pigmentation of cavernicolous forms and of its absence upon that of closely related epigean species. Secondly, a survey of as many epigean forms as are available would help to determine whether any given group of species or genera normally contains any characteristic pigment or pigments. The comparative study of hypogean forms might then reveal the persistence or the loss of particular types of pigmentation. Further, it is now known that many Crustacea depend upon external sources for the development of their own carotenoid pigmentation, and a third line of approach is therefore the investigation of the sources of carotenoid material in the food of cave animals. The present paper is mainly concerned with work along the first and second of these lines.

As material we had at our disposal large numbers of *A. aquaticus*, all of which were collected from a small area on Coe Fen, Cambridge, and identified as

. aquaticus Linn. in the strict sense of Racovitza (1919). This is the dominant pecies in northern Europe generally. Smaller numbers of *A. meridianus* Rac., the ominant species in southern Europe, were obtained from a stream at Vrhnika, ovenia, and the cave-living form *A. aquaticus cavernicolus* Rac. from the Postumia rotte, northern Italy.

Most of the work reported here is concerned with pigments of the melanin group d with carotenoid substances. It will be most convenient if these are discussed parately.

Although the term 'melanin' is familiar to biologists interested in many different anches of the subject, it is not possible to give any satisfactory definition of the rm on chemical lines. The well-known work of Raper (Raper, 1928; Raper & ormall, 1923) on melanin formation and the more recent observations of Pryor 939) are valuable contributions to our knowledge of these pigments, but for esent purposes we must be content to use the term in the sense of Verne (1926): On entend par mélanines des pigments apparaissant dans l'organisme des Vertébrés des Invertébrés, dans des conditions normales ou pathologiques caractérisés ar leur couleur noire ou brune, leur existence sous forme de grains, leur grande sistance à divers solvants et réactifs chimiques.' On account of their chemical ertia these substances do not lend themselves well to chemical investigation.

In the carotenoids, however, we have a group of substances which have been uch studied in recent years and about the chemistry of which a great deal is coming known. They are, moreover, pigments to whose study the elegant methods partition between organic solvents and chromatographic adsorption can be plied with particular convenience and success.

We have in the main followed the standard procedures described by Zechmeister 1934), and Zechmeister & Cholnoky (1937). The pigments were extracted with uitable solvents, transferred to petrol ether (b.p. 50–60° C.) and submitted to artition between this solvent and 90 % methanol. After saponification the pigments resent in the petrol phase were recovered in petrol ether, and a second partition gainst 90 % methanol was carried out. We thus obtained three main fractions, each f which was subsequently recovered in pure petrol ether. These fractions comprised (*a*) xanthophylls originally present in the free state, extracted by methanol t the first partition, (*b*) xanthophylls originally present in the esterified state, xtracted by methanol at the second partition, and (*c*) carotenes, remaining hroughout in the petrol ether phase. After purification and recovery in petrol ether, these fractions were further examined chromatographically, the arsenic-free magnesium oxide, of British Drug Houses, being the adsorbent most frequently employed. Our columns usually measured about 10 × 1 cm. The pigments were subsequently eluted and examined spectroscopically in suitable solvents with the aid of a Hartridge reversion spectrometer fitted with a symmetrical slit. The greatest probable error of this instrument was estimated at $\pm 2\mu\mu$ approx.

As is well known, considerable discrepancies appear between the positions of the absorption maxima as determined by different instruments, and Smith (1936) n particular points out that the best instrument for making these measurements is

a good spectrophotometer. We, however, did not have a suitable machine at our disposal, and preferred to use the less laborious Hartridge apparatus and to compare our readings with those obtained using standard specimens of known pigments prepared from other sources. The standard pigments most generally used in the present work were cryptoxanthine and zeaxanthine, prepared from maize by an adaptation of the technique of Strain (1938), and β-carotene prepared from tomato pulp by the general procedure outlined by Zechmeister (1934).

Use was also made of the technique of mixed chromatography (cf. Lederer, 1938), and our standard preparations were of great value here also. Preliminary experiments using these standard preparations were carried out and it was found that, if the chromatograms were developed by means of 2% methanol in petrol ether, the bands appeared very rapidly, were very narrow and deep in colour, and could easily be recognized by their characteristic behaviour. Thus β-carotene gave a characteristic brown, very narrow band, cryptoxanthine an orange-yellow band, somewhat more diffuse, and zeaxanthine a yellow and relatively very diffuse band. Development with petrol ether alone or with petrol ether containing a small proportion of benzene is a more usual procedure, and is, indeed, most suitable when it is desired to separate pigments from each other for subsequent spectroscopic examination, but it has the disadvantage of being a somewhat lengthy process which uses considerable amounts of solvents. Our method is economic of solvents, is rapid and very convenient, and in our experience is very reliable when standard pigments are available for comparison with the unknowns.

MELANIN IN THE HYPODERMIS OF *ASELLUS* SPP.

The hypodermal pigment of *A. aquaticus* is responsible for the yellow-brown external colour of this species and appears to be a melanin, as stated by Kosswig (personal communication). The following observations support this view. After the animal has been ground to a fine paste and all carotenoid material extracted, a purplish paste remains, and from this no further pigment can be extracted with ethanol, methanol, ether, petrol ether, benzene, chloroform, carbon disulphide, pyridine or trichloracetic acid. Nor is the hypodermal pigment extracted from intact specimens after several years' preservation in alcohol or formalin. The pigment, whether in the intact animal or in the extracted paste, is bleached by hydrogen peroxide in acid solutions, and can be extracted by treating the paste with boiling alcoholic potash to give a deep red solution. Microscopic examination shows that the pigment is present in the form of fine granules within branched chromatocytes and we are indebted to Dr Mark Pryor for informing us that these granules reduce silver nitrate. In all these respects, therefore, the hypodermal pigment of *A. aquaticus* behaves like a typical melanin (see Verne, 1926).

A. meridianus is as deeply pigmented as *A. aquaticus*, but the body colour of *A. aquaticus cavernicolus* is very variable. The hypodermal pigment of all three species is present in the form of a granular deposit enclosed within branched chromatocytes and is not dissolved by toluene, cresol or alcohol over a period of years. It is highly probable therefore that melanin is present in all three species.

EFFECT OF LIGHT ON THE MELANIN OF *ASELLUS* SPP.

If the characteristic paleness of cavernicolous forms of *Asellus* were due to a enotypic suppression of pigmentation associated with the absence of light, we uld expect to find a uniform rather than a variable degree of paleness within a en population. The inheritance of the hypodermal pigmentation has been studied Kosswig (1935) using *A. aquaticus cavernicolus* from northern Italy. He finds t in this species the pigmentation varies in different populations from a depth of our equal to that of normal epigean specimens of *A. aquaticus* to an almost com- te lack of pigment. His breeding experiments demonstrate segregation of aracters determining pigmentation; thus a considerable range of pigmentation was served in the offspring from a cross of two almost unpigmented individuals.

The same author has also stated that light is without influence on pigmentation, d this appears to be the case from our own observations also. Five very darkly gmented specimens of *A. aquaticus* were kept in total darkness for a period of $7\frac{1}{2}$ nths, but no evident reduction in colour took place during this time. During the th month, moreover, one specimen was found to be bearing eggs, and at the end the period the young derived from these eggs appeared darker than most young *aquaticus* of similar age, and might well have attained as deep a colour as the rent had they reached adult size. Large numbers of this species have been kept in e dark for periods of several weeks or months on a number of occasions, and in ery case the young produced appeared to be normal in colour while that of the ults underwent no evident diminution. It appears therefore that the absence of ht for periods of many months has no effect on the persistence of the external loration of adult specimens, nor on the origin of the colour in specimens kept darkness throughout their embryonic development and for the early part of their bsequent free life.

In view of these results we must conclude that it is very doubtful whether the leness observed in many populations of *A. aquaticus cavernicolus* can be ascribed the phenotypic effect of the darkness which characterizes the cave environment. he reduced pigmentation of the cave variety must probably be due to another ctor and is more probably a permanent element in its genetic constitution (see osswig, 1935, 1937; Kosswig & Kosswig, 1936).

The value of Kosswig's work lies in the establishment of the occurrence of gregation of characters in *Asellus* but, as he himself states, the kind of segregation too complex for Mendelian analysis at the present time. We have obtained evi- ence which suggests that an extension of Kosswig's genetical work would be of nsiderable value, for it was found possible to cross *A. aquaticus* with *A. aquaticus vernicolus* with the production of live offspring. The male in the cross was an most colourless specimen of the cave variety, and was collected from the Postumia rotte and brought back to England in a thermos bottle with the addition of ice uring the journey. The female was an English specimen of *A. aquaticus* Linn. It ppeared that the possibility that the female was already fertilized was excluded for ne following reasons. Eight females, including the one eventually used in the cross,

were placed in a dish in October 1938, and by the following January five specimens had died without having produced eggs. In late February two specimens were bearing eggs the development of which had progressed considerably, and the remaining two were not. Of the two remaining specimens one was used for crossing at the end of March and the other was found still to be without eggs in the middle of July. When the male and female were placed together pairing took place at once, and it may be pointed out that, according to Unwin (1919), pairing does not take place between a male *A. aquaticus* and a female of the same species if she has already been fertilized. We may therefore reasonably regard the instant pairing which took place in our experiment as some confirmation of the unfertilized state of the female. In July 1938, one young specimen, pale in colour, was found in the jar, and this died at the age of 40 days, by which time it had attained a fair degree of pigmentation.

Kosswig's theory of the origin of the lack of pigmentation in cave forms involves the spread of loss mutations in the population and may be briefly stated as follows. In the darkness of a cave colour has no apparent significance and may be regarded as of neutral survival value. Since loss mutations are more frequent than positive ones it may be assumed that random mutations for loss of colour will gradually spread through a population, unchecked by any counter-selection. In the initial stages of this process we should expect to find a variable colour in the population and in the final stages a uniform absence of pigment. Kosswig himself has observed both variable populations of *A. aquaticus cavernicolus*, as in the Postumia Grotte, and uniformly unpigmented populations, as in the Grotta di Trebiciano. Our observation that viable young can be produced by crossing the cave variety with the closely related *A. aquaticus*, which is epigean, shows that it may be possible to evaluate the characters determining colour in the epigean species by suitable genetical studies, and to state which specific colour factors have been lost in the cavernicolous form in order to gain some insight into the course of evolution of the cave type.

CAROTENOID PIGMENTS IN *ASELLUS AQUATICUS*

In order to clear the guts of extraneous carotenoid materials, which a preliminary examination of the faeces showed to be present in considerable quantities,[1] the animals were fed on grains of soluble starch, a carotenoid-free diet which was readily taken. This diet was given for at least a week, after which the guts of all specimens examined appeared to contain nothing but starch. The animals were kept in the dark during this preliminary clearance period in order to preclude algal growth, since we found that the green algae which develop in river water exposed to sunlight for several days in the laboratory are relatively rich in carotenoid substances. After this preliminary treatment, some 1500 specimens of *A. aquaticus*, weighing 48 g., were extracted with successive small quantities, first of methanol and then of petrol ether. The pulverized residues were dark purplish red in colour (see p. 138), and this

[1] Chlorophylls *a* and *b*, at least three xanthophylls and a carotene could be detected by use of the micro-procedure of Kuhn & Brockmann (1932).

oloration is apparently due to a melanin. Kaulbersz (1913) had already noticed at this animal develops a pink colour after death, and we have examined a number recently dead, pink specimens separately and found that the nature of their rotenoids is the same as that of fresh specimens but that the reticulated melanin posits appeared to have disintegrated. The reddish colour is evidently due to elanin rather than to the production of any new carotenoid.

The extracts were combined and the pigments quantitatively transferred to trol ether by the addition of water. The resulting deep yellow solution of carote- oid pigments in petrol ether was submitted to partition against 90% methanol. he hypophasic layer contained very little pigment indeed; after transference to chloroethane and adsorption on magnesium oxide two very small bands were btained, indicating (probably) the presence of traces of free xanthophylls in the riginal material. This fraction was too small for further study.

The main epiphasic fraction was saponified in the usual way and, after recovery petrol ether, a second partition against 90% methanol was carried out. The igments remained entirely epiphasic, however. Generally speaking, such a result ould be taken as showing that no xanthophyll esters were present in the original aterial since, after saponification, the xanthophylls would pass into the alcoholic yer at this second partition. As a rule such a conclusion is perhaps justified, since he most commonly occurring xanthophylls are those containing at least two coholic groups and these are wholly hypophasic in the partition test. But the ajority of workers appear to have neglected the fact that monohydric xanthophylls uch as rubixanthine and cryptoxanthine are wholly epiphasic under these conditions nd thus resemble the esterified xanthophylls and the carotenes proper. They may, owever, be distinguished from the carotenes by the fact that they are in part ypophasic with respect to 95% methanol; thus Zechmeister (1934) has written of ryptoxanthine and β-carotene: 'bei der Verteilung zwischen Benzin und 90 proz Methylalkohol suchen beide Farbstoffe die Oberschicht auf; verwendet man jedoch 95 proz Methanol, so wandert das Kryptoxanthin deutlich nach unten. (Abweichung von β-carotene.)' In the present case a part of the saponified pigment passed nto 95% methanol from petrol ether, indicating the presence of a monohydric xanthophyll or of some material behaving in the same manner.

The pigments of the main saponified epiphasic fraction were accordingly separated chromatographically on magnesium oxide, the column yielding three well-defined bands on development with pure petrol ether; these were (1) an orange region 15 mm. deep at the top of the column, (2) a very pale yellow band 3 mm. deep, and (3) a second orange region 10 mm. deep below. These were eluted and examined further.

Band 3. The pigment of this band was wholly epiphasic to 90% and to 95% methanol and therefore appeared to be a carotene. The spectroscopic data of Table 1 were obtained. Rechromatographed on magnesium oxide a single brownish band was obtained; a precisely similar band was obtained with our standard sample of β-carotene and another with a mixture of this with the *Asellus* sample. It may therefore be concluded that band 3 corresponds to β-carotene.

Table 1. *Values for β-carotene*

(All values in $\mu\mu$)

Solvent	Maxima observed	Kuhn & Brockmann (1932)	Smith (1936)	Standard sample
Petrol ether	452; 486	452; 484		452; 485
Carbon disulphide	486; 516		486; 517	

Band 2. The pigment of this band was wholly epiphasic to 90% and 95% methanol but too small in amount for further examination.

Band 1. The pigment of the uppermost band was almost wholly epiphasic to 90% methanol but a part passed readily into 95% methanol. This behaviour strongly suggested that we had here to deal with a monohydric xanthophyll. The epi- and the hypophasic portions gave the same absorption maxima, indicating that we were in all probability concerned with a single substance rather than with a mixture of two substances which happened to behave in a similar manner on the column. Rechromatographed on magnesium oxide and separated from a faint band which appeared below it (? neocryptoxanthine—cf. Zechmeister & Tuzson, 1938) the pigment again gave the same absorption maxima, which agreed well with those of cryptoxanthine.

Since, however, the maxima for cryptoxanthine, and β-carotene are very similar, and since the differences in the phase test alone are not very convincing evidence, we compared the behaviour of our material on a magnesia column with that of standard samples of cryptoxanthine, zeaxanthine and β-carotene. The *Asellus* material gave a band which differed sharply from those of zeaxanthine and β-carotene but was indistinguishable from that given by cryptoxanthine. Mixed chromatograms were also prepared and whereas mixtures of the *Asellus* pigment with β-carotene and zeaxanthine gave rise to a pair of bands in each case, mixtures with cryptoxanthine gave a single band only.

Table 2. *Values for cryptoxanthine*

(All values in $\mu\mu$)

Solvent	Maxima observed	Kuhn & Grundmann (1933)	Standard sample
Petrol ether	451; 485	452; 485·5	451; 485·5
Carbon disulphide + trace ethanol	482; 518	483; 519	486; 516·5
Chloroform	463; 497	463; 497	
Ethanol	454; 487	452; 486	

Finally, the remainder of the *Asellus* pigment was dissolved in methanol and freed from a quantity of white, crystalline material by the addition of enough water to bring the concentration of methanol to 85%. After removal of the precipitate by centrifugation, the pigment was transferred to petrol ether and re-extracted with successive small quantities of 95% methanol until the petrol layer was nearly colourless. The relatively pure pigment present in the alcoholic extracts now gave

spectroscopic data of Table 2.[1] It may therefore be concluded that band 1 rresponds to cryptoxanthine.

It is still not clear, however, whether this pigment, which so far as we know has t hitherto been reported from animal materials, was originally present in the free in the esterified condition, since the behaviour of its esters in the phase test and adsorption columns has not been investigated. It is likely that esters would emble the free xanthophyll rather closely.

SUMMARY

1. The widespread reduction of pigmentation found in cavernicolous animals is scussed.

2. The externally visible coloration of *Asellus aquaticus* Linn., *A. meridianus* ac., and *A. aquaticus cavernicolus* Rac., appears to be of the melanin type.

3. The melanin content of *A. aquaticus* is not appreciably affected if this animal kept in the dark for periods of several months. Offspring produced during this riod are normal in colour.

4. Fertile young resulted from a cross between a colourless female *A. aquaticus vernicolus* and a normally pigmented male *A. aquaticus*. This observation is obably of significance for the genetical analysis of the colour types found among e hypogean asellids.

5. The predominant pigments of *A. aquaticus*, apart from melanin, are β-carotene d cryptoxanthine. Other carotenoid pigments are present only in traces.

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[1] In ethereal solution our product gave no blue coloration with concentrated hydrochloric acid.

THE PIGMENTATION OF CAVERNICOLOUS ANIMALS

II. CAROTENOID PIGMENTS IN THE CAVE ENVIRONMENT

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INTRODUCTION

The present paper records the results of some analyses of carotenoid material in cave detritus, carried out as part of a research on the causes of pigment lack in the cave fauna. There is reason to suppose that the ancestral pigment of the numerous unpigmented cavernicolous Crustacea was the carotenoid pigment astacin, since closely related above-ground species frequently contain this pigment. Thus the amphipods *Orchestia gammarellus* and *Gammarus marinus* have a wholly carotenoid pigmentation of astacin type, while *Gammarus pulex* is believed to contain astacin (Sörensen, 1936). It is a well-known fact that the pigment of many Amphipoda is soluble in the alcohol in which the animals are preserved, and we are justified in concluding that astacin-like pigments are typical of the group. It is unlikely that Crustacea can synthesize carotenoid pigment from non-carotenoid food (*vide* the discussion in Verne, 1926), and the present work forms a preliminary survey of the extent to which carotenoid-containing food is available to the cave fauna.

Several modes of entry of food into caves have been noted. The excreta of bats, on which colonies of coprophagous beetles live, is of minor importance. Accidental entry of leaves, twigs, etc., at the mouth of the cave, does not contribute to the food of animals in the main region of the cave. Detritus carried in by underground rivers is the chief source of food for cave animals, and as shown here, carotenoid material is found in it in some quantity. *Proteus*, which is associated with subterranean rivers, was found to contain some carotenoid material. In the upper regions of caves drip pools are found, fed only by water percolating through the limestone. Detritus from such pools was found to be devoid of carotenoid material, and specimens of the cavernicolous amphipod *Niphargus* from such a pool were found to contain no carotenoid pigment.

TECHNIQUE

Samples were extracted with acetone, methanol, and in some cases subsequently with ether, or petrol ether (b.p. 50–60° C.). Analysis for carotenoid pigments was carried out on the basis of the usual micro-technique (Kuhn & Brockmann, 1932). The extracted pigment, dissolved in petrol ether, was saponified by adding an equal

lume of 5% potash in absolute alcohol and maintaining the mixture at 40° C. at least 3 hr. Pigment was regained in petrol ether by the addition of water, and rtitioned between petrol ether and 90% methanol, the carotene remaining in the piphasic) petrol ether and the freed xanthophylls passing into the (hypophasic) ohol layer. The carotene and xanthophyll fractions were transferred to pure trol ether and then subjected to chromatographic analysis by adsorption on umns of adsorbent material about 1 cm. in width and 8 cm. in height. The re-tant coloured bands were eluted and their absorption maxima determined for mparison with the absorption maxima of known carotenoid pigments. As an ditional check, the carotenoid nature of the pigments was usually confirmed by atment of the dry chloroform solution of pigment with a solution of antimony chloride in chloroform, a blue colour being consonant with the presence of rotenoid.

In the experimental section below, the use of the terms 'epiphasic' and 'hypo-asic' without further qualification refers to partition between petrol ether (b.p. –60° C.) and 90% methanol.

EXAMINATION OF MUD FROM CHISLEHURST CAVE FOR CAROTENOID MATERIAL

Some mud from the top half-inch of a deposit on the floor of a gallery in hislehurst Cave, Kent, was collected. The mud appeared to have been washed in om a hole in the roof and to have reached this gallery, which was in total darkness, suspension in water. The sample was examined exhaustively for carotenoids but one could be detected.

The sample, weight 500 g., was extracted exhaustively with acetone and other lvents, and all pigment transferred to 1000 c.c. of ether, a deep red-brown solution sulting. It was observed during the extraction that all solutions had a strong reen-blue fluorescence, that in none of them was an absorption band due to hlorophyll observed in the spectrum, and that when solutions were taken to dry-ess a red-black solid remained.

A portion was chromatographed in solution in petrol ether on a column of agnesium oxide. Two deep brownish diffuse bands appeared at the top of the olumn, and could not be eluted by the usual elution media for carotenoids, e.g. etrol ether containing methanol or benzene. After elution with pyridine, a blood-ed solution resulted, but the pigment had only a single absorption band and re-ponded negatively to the antimony trichloride test. Chromatographing on agnesium oxide serves therefore to remove a great part of the non-carotenoid igments in the extract.

Of the main extract 50 c.c. were examined by the usual micro-method (Kuhn Brockmann, 1932), and separated into carotene, xanthophyll, and xanthophyll-ster fractions. At no stage was pigment observed to be sharply epiphasic or hypo-hasic. The carotene fraction when chromatographed on magnesium oxide gave se to a dirty yellow staining at the top of the column. A small yellow band was

separated from this and eluted through the column by passage of petrol ether + 2% methanol. Another was eluted by passage of pure benzene. The first eluted fraction exhibited an absorption maximum at 456 $\mu\mu$, the second at 459 $\mu\mu$. Neither fraction gave a positive reaction to the antimony trichloride test. The xanthophyll fraction was not appreciably adsorbed when chromatographed in petrol ether on calcium carbonate. The pigment eluted through the column by petrol ether and benzene 1 : 1 showed an absorption maximum at 456 $\mu\mu$ in carbon disulphide, and gave a negative antimony reaction. The xanthophyll ester fraction was chromatographed in petrol ether on calcium carbonate. Any possible carotenoid was eluted through the column with petrol ether and benzene 1 : 1; the elutrate had an absorption maximum at 457 $\mu\mu$ in carbon disulphide, and failed to respond to the antimony test.

In this sample of detritus therefore no trace of carotenoid or of chlorophyll was observed. Large quantities of a non-carotenoid pigment were present, with the following properties. The pigment is fluorescent in solution, and when dry is a red-black solid. The pigment is not sharply epiphasic or hypophasic. During various processes the pigment was found to be insoluble in water, and saturated aqueous salt solution, sparingly soluble in petroleum ether, moderately soluble in methanol, and very soluble in ether, acetone, benzene, chloroform, and dichloroethane. The absorption maximum of the pigment in carbon disulphide is 457 $\pm$ 2 $\mu\mu$. The pigment has not been identified.

DETRITUS SAMPLES FROM THE POSTUMIA GROTTE

The Postumia Grotte of north Italy, formerly known as the Adelsberg Grotto, is the largest cave system in Europe, and contains 27 km. of passages so far explored. The River Piuca which flows through it floods the upper galleries during the winter and leaves pools behind when it recedes. In these pools are found such characteristic cave animals as *Proteus anguineus* Laur, and the decapod crustacean *Troglocaris schmidti* Dorm. In still higher regions of the cave pools of a different ecological status are found—these are never flooded by the river and derive their water from roof drippings. They contain a smaller number of cave animals, notably *Niphargus* spp. Plant remains are commonly found in those pools subjected to seasonal flooding, while in the drip pools only a fine clay is usually found.

Four samples of detritus were collected:

Sample A. Earth from just within the entrance to the Grotto Nera. Fauna, various epigean species.

Sample B. Mud from a small pool in the Gnomo della Grotte, well within the cave. Water in the pool derived solely from roof drippings. Nine specimens of *Niphargus* spp. collected from the pool.

Sample C. Debris, chiefly old leaves and pieces of wood, from the edge of the water at the end of a mud passage in the Grotto Nera, well within the cave. Fauna planarians, *Proteus*, *Troglocaris*, and *Asellus aquaticus cavernicolus*.

Sample D. Mud from the passage described under sample *C*, but above the water level. The area is flooded in winter. Fauna *Titanethes* (an amphibious isopod).

Investigation of sample A

75 g. of fresh sample were extracted with acetone and all pigment transferred petrol ether, 200 c.c. of a fine yellow-brown solution with a greenish fluorescence sulting. Absorption bands approximating to those of chlorophyll were observed the spectrum.

The entire petroleum ether solution was chromatographed on magnesium oxide. he following system of bands appeared:

Orange-brown Green Orange Green Yellow	In top 12 mm. of column
Yellow stain over remainder of column	

A fine yellow solution was eluted through the column by passage of 2% ethanol in petrol ether ('Methanol fraction'). A further quantity of pigment was uted by passage of 2% benzene in petrol ether ('benzene fraction'). Various rown and green bands remaining on the column were rejected as being obviously f non-carotenoid nature.

The 'methanol fraction' was hydrolysed and partitioned. The epiphase gave a rongly adsorbed yellow band when chromatographed on magnesium oxide in olution in petrol ether. Two small pigment fractions were eluted through the olumn by successive passage of 0·2 and of 2% methanol in petrol ether. The two actions were epiphasic to 90 and 95% methanol, had a yellow fluorescence, showed o absorption bands in chloroform, and responded negatively to the antimony richloride test. The hypophase was transferred to dichloroethane and chromatoraphed on magnesium oxide. Three yellow bands appeared and were eluted eparately. The upper band was epiphasic, and probably represented some carotene ot fully extracted by the partition with petrol ether. The middle band was not harply epiphasic. Neither band contained sufficient pigment for further analysis. he lower band was chiefly hypophasic; the spectrum showed absorption maxima t 508 and 480 $\mu\mu$ in carbon disulphide. A xanthophyll was therefore present.

The 'benzene fraction' was saponified and chromatographed in petroleum ther on calcium carbonate. A yellow stain appeared throughout the column, and brown band at the top. The two regions were eluted separately. The pigment rom the brown band responded negatively to the antimony trichloride test and xhibited no absorption bands. The pigment from the yellow staining over the olumn was almost wholly epiphasic to 90 and 95% methanol. In chloroform, the pectrum showed sharp absorption bands at 503 and 473 $\mu\mu$.

Nature	Observed absorption maxima	Solvent	Cp. literature
Carotene	503 473 $\mu\mu$	$CHCl_3$	503 470 (γ-carotene)
Xanthophyll	508 480 $\mu\mu$	CS_2	508 475 (lutein)

At least two carotenoid pigments were therefore present in small quantity in sample A, as summarized in the previous table. The maxima recorded for γ-carotene are from Winterstein (1933), and those for lutein from Kuhn *et al.* (1931).

Investigation of sample B

240 g. of fresh sample were extracted with acetone and the pigment taken up in petrol ether. [During the washing of the petrol ether with water, much brownish non-carotenoid matter separated. It was readily soluble to give a deep red solution in ether.] The extract was chromatographed in petrol ether solution on magnesium oxide. A variety of orange, yellow, and brown bands appeared. All possible carotenoid pigment was eluted through the column with 2% methanol in petrol ether and 2% benzene in petrol ether. The remaining brownish bands, non-carotenoid in nature, were discarded.

The elutrate was hydrolysed and partitioned. A deep yellow epiphase and a pale yellow hypophase resulted. The epiphase was chromatographed in solution in petrol ether on magnesium oxide and developed with 0·5% methanol in petrol ether. A brown band was left at the top of the column and discarded. An orange band below it was eluted (absorption maximum in carbon disulphide 453 $\mu\mu$).

The third and lowest band was diffuse yellow, with an absorption maximum at 456 $\mu\mu$. Neither of the two lower bands responded positively to the antimony trichloride test. The *hypophase* when chromatographed in solution in petrol ether on calcium carbonate gave only a diffuse yellow staining. The pigment when eluted had an absorption maximum at 455 $\mu\mu$ in carbon disulphide, and responded negatively to the antimony trichloride test.

In this relatively large sample therefore carotenoid pigments and chlorophyll were absent. Further, the nine specimens of *Niphargus* collected from the water yielded no trace of coloration when extracted in the usual way, even when any possible pigment was concentrated in a minute volume of petrol ether.

Investigation of samples C and D, and comparison of the four samples

For comparative purposes, small quantities (30 g.) of each sample were examined under comparable conditions. They were extracted exhaustively with acetone, ethanol and ether, and pigment transferred to ether. The ether solutions were dried over anhydrous sodium sulphate, evaporated to dryness under reduced pressure, and the last traces of solvent removed by heating at 70° in a current of coal gas. The residues were weighed, redissolved in ether, and the spectra examined for chlorophyll bands. Finally each ether solution was hydrolysed and analysed for carotenoid material. The residues of extracted detritus were weighed and cooled in a desiccator to constant weight over a period of months. The properties of the samples are compared in the following table:

Colour of ether solution after saponification	Percentage water	Percentage extractable matter in dry weight of sample	Chlorophyll bands
Sample A: Wine red, strong green fluorescence	21	0·25	Present
Sample B: Palish yellow-orange, strong green fluorescence	25	0·04	Absent
Sample C: Yellow-gold, no fluorescence	60	0·73	Present
Sample D: Pale yellow-gold, slight green fluorescence	36	0·02	Present

A large proportion of the non-carotenoid colouring matter was removed by romatographing the hydrolysed pigments in solution in petrol ether on magsium oxide, the carotenoid pigments being recovered by elution of the column th 2% methanol in petrol ether. The eluted carotenoids were hydrolysed anew saponify any remaining chlorophyll or xanthophyll ester, and were examined for rotenoids by the usual Kuhn and Brockmann microtechnique, with the following sults.

Sample A. Various pigment fractions were isolated from the small quantity of mple A used, but none had sharp absorption bands or gave a positive antimony chloride test. It will be recalled, however, that the investigation of a greater uantity of sample A already described had demonstrated the presence of a small uantity of carotene and xanthophyll.

Sample B. As already described, an analysis of 240 g. of this sample showed no ace of carotenoids.

Sample C. After partition, the epiphase had absorption bands at 512 and 485 $\mu\mu$ carbon disulphide. This approximates to the values of 517 and 486 $\mu\mu$ recorded r β-carotene in this solvent by Smith (1936). This pigment and a stock sample of -carotene gave the same behaviour on a magnesium oxide column when chromatoraphed in solution in petroleum ether and developed with 2% methanol in petrol ther. The hypophase gave rise to four yellow bands when chromatographed in etrol ether on a column of calcium carbonate. The upper two bands, indistinctly eparate, were eluted together, and the mixture exhibited absorption maxima at 490 nd 457 $\mu\mu$ in chloroform. The next lowest band showed after elution maxima at 88 and 456 $\mu\mu$ in chloroform. The lowest band was large, and exhibited absorption naxima at 492 and 458 $\mu\mu$ in chloroform, and 505 and 485 $\mu\mu$ in carbon disulphide. 'he absorption maxima for the pigment of the lowest band, and its position on the olumn, suggest a mixture of lutein and zeaxanthin, the two commonest xanthohylls. In actual fact two bands did appear when the pigment of this band was echromatographed in solution in dichloroethane on a magnesium oxide column. 'he three fractions eluted from the chromatogram responded positively to the ntimony trichloride test.

Sample D. Little pigment was present. It distributed itself during partititon ith slightly more pigment in the epiphase than in the hypophase. Insufficient igment was present to give absorption bands or a decisive antimony trichloride eaction. Probably a little carotene and xanthophyll are present.

CAROTENOID PIGMENTS IN THE CAVE SALAMANDER *PROTEUS*

The blind and almost colourless cave salamander *Proteus anguineus* Laur. of north Italy and Yugoslavia is a typical member of the cave fauna. It is always found in association with an underground river, and from the evidence given earlier in this paper it may be assumed to have access to carotenoid-containing food.

Three large entire specimens were extracted by grinding with sand and methanol. Pigment was transferred to petrol ether and separated into carotene, xanthophyll, and xanthophyll ester fractions. The carotene fraction was found to contain the greater proportion of the pigment, and was chromatographed in petrol ether on magnesium oxide. A band of typical β-carotene appearance resulted, which on elution showed absorption maxima at 518 and 489 $\mu\mu$. This agrees fairly well with the values of 517 and 486 $\mu\mu$ recorded for β-carotene in this solvent by Smith (1936). The xanthophyll fraction gave rise to a small yellow band when chromatographed in petrol ether on calcium carbonate, and two rather indefinite absorption maxima could be seen. No trace of xanthophyll ester could be demonstrated.

Pigment from the eviscerated body of two specimens was found to pass almost wholly into the xanthophyll fraction. Pigment from the liver passed almost wholly into the carotene fraction. The guts after cleaning yielded hardly a trace of pigment.

To summarize: the total quantity of carotenoid pigment in *Proteus* is small. The whole body contains carotene (probably β-carotene) and free xanthophyll. The eviscerated body contains chiefly xanthophyll, the liver chiefly carotene, while the gut is almost devoid of carotenoid pigment.

DISCUSSION

In cave mud from both England and Italy large quantities of a non-carotenoid red pigment can be extracted by organic solvents. The pigment has a marked absorption maximum in the spectrum at $457 \pm 2\,\mu\mu$. It has not been identified.

The distribution of carotenoid material in caves is correlated with the detritus carried in by subterranean rivers. In the Postumia Grotte, air-borne detritus from near the entrance contained only a little carotenoid material (sample A). Organic detritus from a pool in connexion with the underground river contained no less than 0·73 % of matter extractable by organic solvents per dry weight of sample, and in the extracted material large quantities of carotenoid material were present, including β-carotene and at least four xanthophylls, together with much chlorophyll (sample C). The temperature of water of caves is as a rule uniformly low (about 10° C.) and has a low oxygen tension: this may account for the preservation of the carotenoids. Fine detritus carried in by the river but exposed to the air by the summer recession of the water contained only a little carotene and xanthophyll (sample D). Sample B on the other hand was from a pool not in connexion with the river, and was found to be devoid of carotenoid material; specimens of *Niphargus* from the same pool were also free from carotenoid. In such drip pools it would seem therefore that the water which feeds them is freed of carotenoid as it percolates

rough the limestone, and that there is no synthesis of carotenoid by bacteria, ngi, or the fauna. Matter extractable by organic solvents was at the low level of 04 % dry weight of sample.

Proteus was found to contain a little carotenoid pigment in the body tissues. nce *Proteus* is a carnivore, its chief article of diet being the cave Crustacea, it may assumed that there is a transference of carotenoid material from detritus to rustacea, and thence to *Proteus*. Cave Crustacea therefore seem to pick up vegetable rotenoid material when it is available. Since, however, the Crustacea are practi- lly colourless and lack the characteristic pigment of epigean forms, it would appear be established that the specific animal pigment astacin is lacking whether or not getable carotenoid material is available in the diet.

In the organic detritus of sample C a preponderance of xanthophyll was demon- rated. Three xanthophyll fractions were isolated with absorption maxima in loroform at 490 and 457 $\mu\mu$, 488 and 456 $\mu\mu$, and 492 and 458 $\mu\mu$. These figures call the findings of Baudisch & von Euler (1934), who found that peat rich in lcium carbonate had a considerable xanthophyll content, the absorption maxima the hydrolysed pigment in chloroform being at 488 and 455 $\mu\mu$. They have ggested the theory that a natural adsorption of xanthophyll takes place on lime- one, vegetable oils acting as solvent, while carotenes are but weakly adsorbed, and the present work there is certainly a preponderance of xanthophyll over carotene organic detritus associated with limestone. In deep-sea mud Fox (1937) found preponderance of xanthophyll, with absorption maxima comparable with those corded by us and by Baudisch and von Euler.

SUMMARY

1. Organic detritus was examined from Chislehurst Cave and from the ostumia Grotte (north Italy). The origin of carotenoid material in the detritus was losely associated with the influence of underground rivers. In the Postumia rotte, large quantities of carotenoid material were present in detritus swept in by ne underground river and deposited in a pool. A smaller quantity was present in imilar detritus which had been exposed to the air. Very little was found in air- orne detritus at the mouth of the cave, exposed to the air, to light and to tempera- ures higher than those prevailing within the cave. No carotenoid pigments were ound in sediment from a drip pool, which was not connected with the underground iver. In Chislehurst Cave no carotenoid pigments could be detected in detritus xposed to the air. These observations indicate that the cave fauna must have some ccess to carotenoid pigments.

2. A carotene (apparently the β-compound) was found, together with free anthophylls in the cave salamander *Proteus*. The distribution varied in different arts of the body. No carotenoids were detected in the cave amphipod *Niphargus*, aken from the drip pool mentioned above.

3. In the carotenoid materials of detritus, xanthophylls predominate. This has een observed by other workers in carotenoid pigments from peat associated with

calcium carbonate. The absorption maxima of the xanthophyll isolated by these workers agree closely with the maxima found in the present investigation.

4. An unidentified red pigment with a powerful fluorescence was found in all the detritus samples studied. It was soluble in organic solvents and showed absorption maximum at $457 \pm 2\mu\mu$ in carbon disulphide.

The above investigations have been carried out during the tenure of a Maintenance Grant from the Department of Scientific and Industrial Research. The work has been supervised by Dr E. H. F. Baldwin, to whom I am indebted for advice. I am especially indebted to Mr R. S. Hawes, B.Sc., for his careful collection and transmission to England of the samples from the Postumia Grotte.

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THE EFFECT OF IONIC COPPER ON THE OXYGEN CONSUMPTION OF *GAMMARUS PULEX* AND *POLYCELIS NIGRA*

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(With Five Text-figures)

INTRODUCTION

The oligodynamic action of copper and certain other heavy metals is a somewhat mysterious chapter in physiology. Many workers have found that solutions of copper, silver, mercury and gold are toxic to bacteria and fresh-water animals and plants at dilutions that are almost fantastic; to cite some of the writer's results, 4×10^{-6} *N* solution of copper nitrate is fatal to *Gammarus pulex* in less than 4 hr., 4×10^{-4} *N* solution fatal to *Polycelis nigra* in $6\frac{1}{2}$ hr., and a 3×10^{-5} *N* solution fatal to toad tadpoles in 2 hr. The toxicity of silver and mercury is even more remarkable; 10^{-5} *N* $AgNO_3$ kills *Polycelis* in 30–40 min., and $HgCl_2$ is fatal to this animal at 10^{-6} *N*, 1 g. Hg in 10,000,000 c.c. water. No satisfactory explanation has been advanced for the high toxicity of these metals at great dilutions, and there has been some discussion as to whether these metals exert their poisonous effect in the colloidal or the ionic state. Earlier workers seem to have favoured the former alternative, and in support of this is the fact that colloidal gold and silver have been employed as germicides with some success. On the other hand, Freundlich & Söllner (1928) have shown that when silver foil is kept in distilled water for 3 days 3×10^{-5} g./l. of silver goes into ionic solution, and that the poisonous effect of this solution is imitated by a silver nitrate solution of equivalent concentration. Leitner (1930) also concluded that the oligodynamic metals acted in ionic form. According to one theory, discussed by Seifriz (1936, p. 119), bacteria are killed because they become coated with a layer of metal; while Heilbrunn (1928, p. 144) states that solutions of $CuCl_2$ in sea water effect a coagulation of the protoplasm of sea-urchin eggs, clearly shown by centrifuge tests, even at a dilution of 10^{-5} *M*.

Voegtlin *et al.* (1925), observing that copper sulphate and gold chloride reacted with reduced glutathione, suggested that the oligodynamic action of these metals might be due to disturbance of the glutathione equilibrium, and that the death of the organism 'might be conceived as a special type of asphyxia'. It is now believed that copper is one of the metals whose catalytic action is an essential feature of the glutathione system, though whether this is so in the case of the organisms to which the metal is so highly toxic is uncertain. Meldrum (1934, p. 40), remarking that

nothing is known of the mechanism of oligodynamic action, also puts forward the suggestion that it is exerted by the inactivation of catalytic mechanisms, and cites the work of Wieland & Mitchell (1932) who have shown that Cu, Ag, Au and Hg retard the enzymic dehydrogenation of xanthine. Cook (1926) states that copper chloride solutions cause a rapid decline in the respiration rate of *Aspergillus* and *Nitella*. The list of substances whose toxic action is due to their power of reducing or inhibiting cellular respiration is a growing one; HCN, H_2S, CO and iodoacetic acid have been recognized as respiratory inhibitors (the action of the first three is fully discussed by Meldrum, 1934), and Keilin (1933, 1936) has shown that sodium azide has a physiological action very similar to that of cyanide. The possibility that the oligodynamic action of the heavy metals may be due to the inactivation or destruction of substances essential for the maintenance of cellular respiration thus appeared worthy of investigation, and prompted the present study.

EXPERIMENTS WITH *GAMMARUS PULEX*

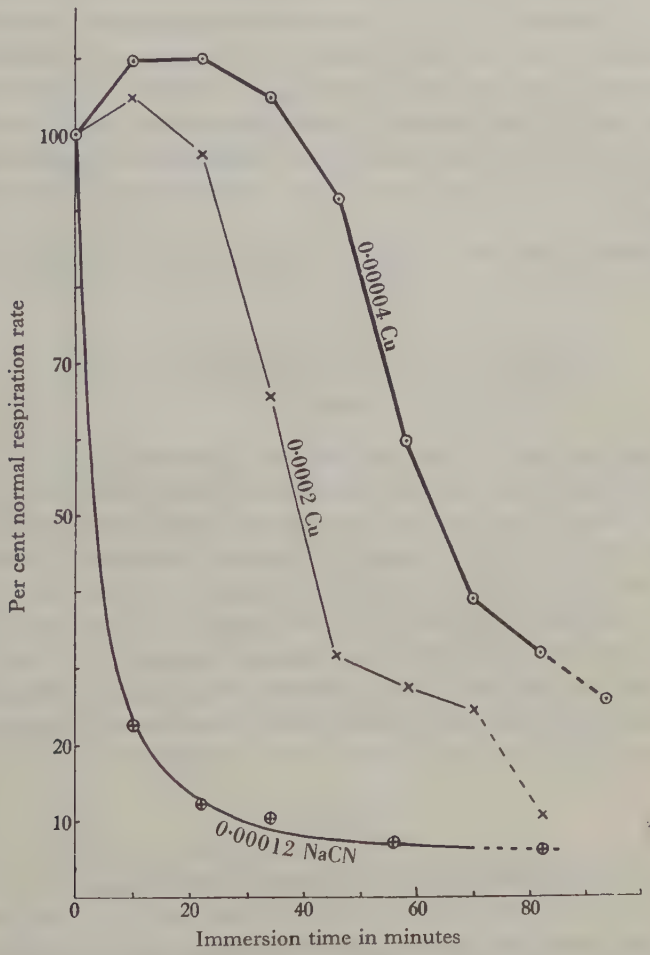
Method

The technique employed for observing the changes in respiration rate occurring during the survival time will be understood from the following description of a typical experiment with 4×10^{-5} *N* $CuSO_4$. Fifteen animals were placed in a 70 c.c. bottle and after two preliminary rinsings this was filled with tap water of measured oxygen content and closed with a perforated stopper. After 15 min. a sample of water was drawn off, its oxygen content measured, and the normal oxygen consumption of the animals thus determined. Two or three successive experiments usually gave results which did not differ by more than 2–3 %. The bottle was then filled with the copper solution, 10 min. later a sample was withdrawn and after two rinsings the bottle was refilled with fresh solution. In this way the oxygen consumption of the animals was measured every 12 min., 2 min. of each interval being occupied with drawing off a sample, rinsing and refilling the bottle.

The animals remained in the bottle for the whole of the experiment. Filling the bottle, and drawing off a sample, took about 15 sec. To ensure consistency the filling of the bottle was started at the beginning of the 10 min. interval, and the withdrawal of the sample completed at the end of the 10 min. Water, solutions, and the bottle containing the animals were maintained at 18° C. in a thermostatically controlled electrically heated oven. In water, and during the early part of the survival time in copper solutions, the animals swam continuously and no agitation was necessary; towards the end of the survival time swimming power was lost and the bottle was gently agitated every 2 min. to avoid stratification. The time when the animals began to die (indicated by the cessation of pleopod movement) was observed, and the experiment was discontinued when half were dead.

Oxygen concentrations were measured by the Winkler method, using *N*/56 thiosulphate measured with a 5 c.c. micro-burette graduated in 1/100ths of a c.c. The presence of copper salts introduces a slight error into the Winkler method. At 0·01 *N* $CuSO_4$ this error is about 2 %, and with decrease in concentration it

idly becomes inappreciable. The water used for the determination of the normal piration rate, and for the solutions, was Aberystwyth tap-water slightly under-urated with air at 18° C. In no experiment was the oxygen content of the water olution permitted to fall to less than 75 % saturation.



. 1. The effect of 0·0002 and 0·00004 *N* $CuSO_4$ and 0·00012 *N* NaCN on the respiration rate of *mmarus pulex*. Each plotted point records the respiration rate for the preceding time interval. here the graphs become dotted the animals have begun to die. *p*H of copper solutions 6·2 and respectively. *p*H of cyanide solution 6·8. Temp. 18° C.

Results

Representative results obtained with *Gammarus* are set out in Fig. 1. It will be ticed that the first response to the copper sulphate solutions is a slight rise in the spiration rate; this probably results from increased activity, for the animals swim riously during the early part of the survival time. After about 35 min. in the

4×10^{-5} solution, or 25 min. in the 2×10^{-4} solution, the respiration rate falls rapidly, the animals become unable to swim, fall to the bottom of the bottle and continue feeble but regular movement of the pleopods. When the respiration rate is still 25% or more of the normal value it is noticed that some of the animals are quite inert.

A 0·00012 *N* solution of sodium cyanide, fatal in about the same time, rapidly depresses the respiration rate to less than 10% normal and inhibits swimming almost immediately. The respiration rate curve obtained is of quite a different type. Thus unless we are to believe that the copper ions take some time to enter the body, and then effect a sudden and very drastic reduction of the respiration rate, we must conclude that the death of the animal is not brought about by the inhibition of cellular respiration.

No further experiments were carried out with *Gammarus* and work was continued with *Polycelis nigra.*

EXPERIMENTS WITH *POLYCELIS NIGRA*

Method

The technique adopted in experiments with *Polycelis* closely resembled that adopted with *Gammarus*. The number of animals used depended on the time intervals over which the respiration rate was measured. For 12 min. intervals 120–200 animals were necessary to give a reduction of oxygen concentration measurable with sufficient accuracy; for 30 min. intervals seventy animals were used, and for 2 hr. thirty sufficed. When the solutions rendered the animals incapable of ciliary locomotion the bottle was agitated gently at frequent intervals. The experiment was discontinued when the animals began to disintegrate; their disintegration appears to liberate substances into the solution which introduce a large error into the Winkler method, and an apparent rise in the respiration rate.

Results

The effect of $CuSO_4$ solutions over the concentration range 0·01–0·0004 *N* was studied, and a representative series of results is given in Fig. 2. It will be noticed that the 0·004 and 0·001 Cu solutions produce a considerable rise in the respiration rate, which is followed by a rapid decline. The preliminary rise appears to be due to increased activity; when the bottle is filled with tap-water the animals are mostly inactive, the majority crawl some distance up the side of the bottle and then remain still. When the water is replaced by the copper solution ciliary locomotion is instantly and completely inhibited, and the animals are thrown into a state of violent and continuous muscular movement which gradually becomes less energetic as the decline in respiration rate sets in. It appears that the cilia are at first paralysed and later destroyed, for if the animals are removed from a 0·004 *N* solution in 10 min. after immersion, washed, and placed in a Petri dish of tap-water, normal ciliary locomotion is almost immediately resumed; but removal in 25–30 min. results in

ilure to resume gliding and eventual death. A similar rise in respiration rate is aduced by barium chloride, for this salt acts as a powerful stimulant to muscular ovement. A 40% rise in respiration rate can be produced with the animals in p-water if the bottle is agitated sufficiently frequently to keep them in a state of ntinuous activity, but the movement so produced mainly takes the form of active iliary locomotion, not muscular movement.

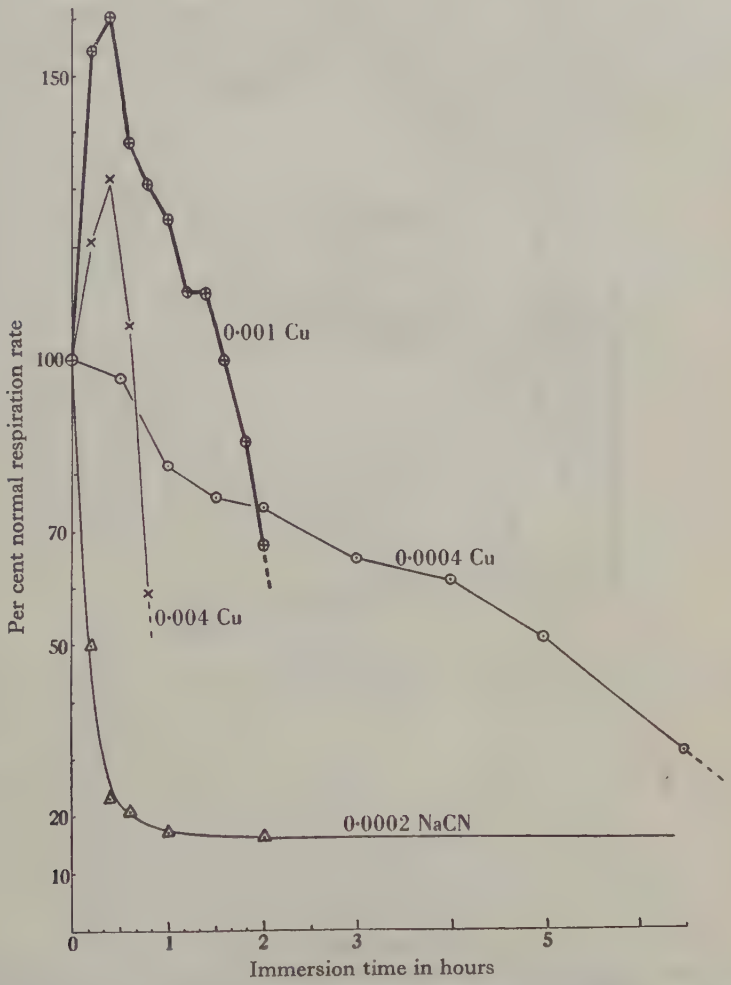


Fig. 2. The effect of 0·004, 0·001 and 0·0004 *N* $CuSO_4$, and 0·0002 *N* NaCN on the respiration rate of *Polycelis nigra*. *p*H of solutions 5·6, 5·8, 6·0 and 6·8 respectively. Other details as Fig. 1.

A 0·0004 *N* copper solution does not inhibit ciliary locomotion; the animals glide slowly or remain quiescent during the early part of the survival time and no increase in respiration rate is observed. Later, as the respiration rate falls steadily, they become more and more inactive and eventually begin to disintegrate.

To see whether the depression of respiration rate caused by the copper solution is sufficient to account for the death of the animals we may compare the effect of

cyanide. The respiration rate curve for 0·0002 *N* NaCN drawn in Fig. 2 shows that in this solution the oxygen consumption rapidly falls to less than 20% of the normal value, whereas in the copper solutions the animals die when it is far above this level. And the cyanide solution is not fatal, the *Polycelis* were practically all alive in 4 days, when their respiration rate was about 16% normal, and were still capable of ciliary

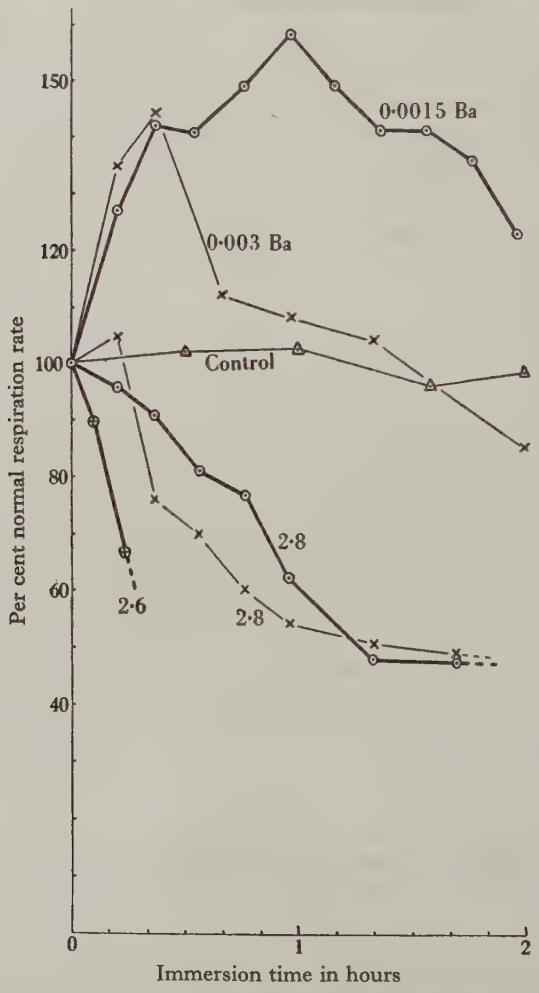
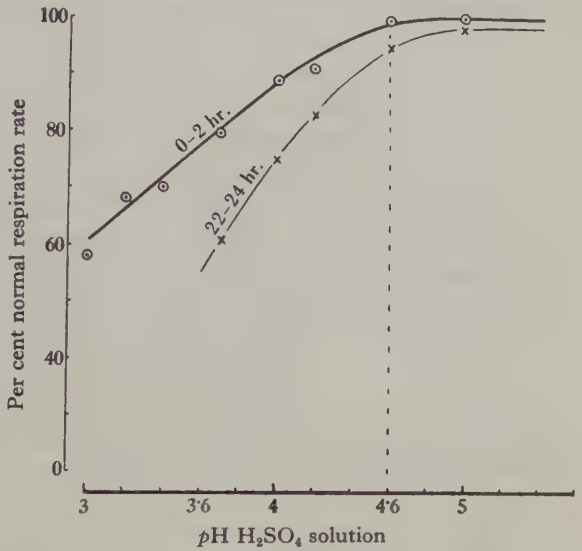


Fig. 3. The effect of 0·0015 and 0·003 *N* $BaCl_2$, and H_2SO_4 solutions of *p*H 2·6 and 2·8 on the respiration rate of *Polycelis nigra*. Two experiments with H_2SO_4 *p*H 2·8 are recorded. *p*H of barium chloride solutions 6·4. Other details as Fig. 1.

locomotion though their speed of gliding was reduced from the normal value of 1·7 mm./sec. to 0.5–0.8 mm./sec. The results thus appear to indicate that the death of the animals in the copper solutions does not result from the inhibition of cellular respiration, and that the depression of respiration rate observed is merely a symptom of the toxic process, not its essential feature. In a brief review of the physiological effect of toxic copper solutions Mitchell (1938, p. 296) concludes that the high

xicity of copper salts rests in the activity with which they combine with proteins, d that their penetration into living cells results in the precipitation of the cyto- asmic proteins as copper proteinates, and a gradual complete destruction of the tire protoplasmic structure. The writer's results appear to fit into this picture asonably well.

Copper sulphate solutions are acid as a result of hydrolysis. The effect of drogen ions upon the respiration rate of *Polycelis* is shown in Figs. 3 and 4. It ill be seen that hydrogen ion concentrations that are speedily fatal (2·6, 2·8) bring out a sharp decline in the respiration rate, that solutions of *p*H 3·0–4·0 have less



g. 4. The effect of hydrogen ions (H_2SO_4 solutions) on the respiration rate of *Polycelis nigra*. -2 hr., average respiration rate for the first 2 hr. immersion. 22–24 hr., average respiration rate for e 22nd–24th hour. Temp. 18° C.

fect, and that a *p*H of 5·0 has little action even in 24 hr. Very acid solutions are tal too rapidly for the decline in respiration rate to be followed. This result is in greement with the observations of Campbell (1923) who found that the injection f HCl reduced the metabolism of anaesthetized cats, and those of Haldane & Vigglesworth (1924) who state that the acidosis resulting from the ingestion, by nan, of large doses of ammonium chloride seems to result in depression of the xygen consumption. The *p*H of the most acid of the copper solutions used in the xperiments with *Polycelis* was 5·6, and thus the hydrogen ions set free by hydrolysis o not appear to be responsible for the changes in respiration rate observed, nor the acidity of the solution responsible for the inhibition of ciliary locomotion for a *p*H of 4·4 H_2SO_4 has no apparent effect on the ciliary locomotion of *Polycelis*. t *p*H 4·0 gliding becomes erratic and intermittent, and at *p*H 3·4 the cilia are aralysed.

This, however, is not the only way in which hydrogen ions may enter into the icture. When heavy metal salts come into contact with living protoplasm the

precipitation of proteins by the cation results in the formation of free acid, and part of the salt's poisoning effect (at high concentrations, at least) takes the form of corrosion by the acid so formed. The nitrates of the heavy metals have a marked corrosive as well as astringent action, as their reaction with proteins liberates the highly ionized nitric acid; the acetates, citrates and tartrates, on the other hand, liberate weak acids and their corrosive is much less pronounced. (For a full discussion see Edmunds & Gunn, 1936, pp. 111–14.) Exactly how far the toxic action of heavy metals at great dilution is due to the formation of free acid within the living cells of the organism is another question which cannot be answered without further experimental evidence.

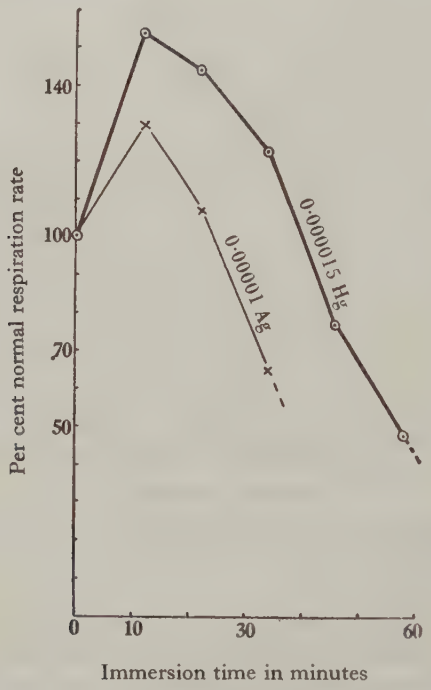


Fig. 5. The effect of 0·00001 $AgNO_3$ and 0·000015 $HgCl_2$ on the respiration rate of *Polycelis nigra*. *p*H of both solutions 6·6. Other details as Fig. 1.

The effect of other oligodynamic metals

Experiments with gold chloride were not successful, as the presence of this salt appears to introduce a very large error into the Winkler determination. Experiments with silver nitrate (in which glass-distilled water was used) and mercuric chloride yielded results essentially similar to those obtained with copper sulphate. Two representative results are given in Fig. 4; in each case the salt immediately inhibits ciliary locomotion and induces a preliminary phase of active muscular movement, disintegration of the animals beginning when the oxygen consumption falls to about half the normal value.

SUMMARY

The suggestion has been put forward that the oligodynamic action of certain avy metals is the result of the destruction or inactivation of substances essential cellular respiration. In a study of the effect of copper sulphate solutions on the ygen consumption of *Polycelis nigra* it is found that solutions of concentration 001–0·01 *N*, fatal in 2 hr. or less, induce a marked preliminary rise in the respira-n rate; this appears to be due to the inhibition of ciliary locomotion and increased iscular activity. A similar increase is produced by increasing the activity of the imals by mechanical means, or by a muscle stimulant (barium chloride). Over latter part of the survival time the respiration rate drops rapidly and disintegra-n of the animals begins when it falls to about 60% of the normal value. A 0·0004 *N* pper sulphate solution does not inhibit ciliary locomotion, does not stimulate uscular activity, and the oxygen consumption undergoes a steady decline. 0·0002 *N* NaCN solution rapidly depresses the respiration rate to less than 20% the normal value, but is not fatal, the animals surviving over 4 days. Hydrogen ns, at the concentrations resulting from the hydrolysis of the salt, have no appre-able effect on the oxygen consumption, but at lethal concentrations (*p*H 2·6, 2·8) ect a speedy depression. The results suggest that the depression of respiration te observed is insufficient to account for the death of the animals, and is no more an a symptom of the toxic process.

A similar general result was obtained in experiments with silver nitrate and ercuric chloride, and also in experiments on the comparative effect of copper lphate and sodium cyanide solutions on the oxygen consumption of *Gammarus ulex*.

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THE ROLE OF THE PITUITARY AND THE THYROID IN THE DEVELOPMENT OF TELEOSTS

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(With One Plate and One Text-figure)

INTRODUCTION

External appearance undergoes a striking transformation at a certain stage in the life cycle of some teleostean fishes. The previous pigmentary pattern of the body is replaced by a brilliant silveriness. Examples are the change from the parr to the smolt condition of *Salmo* and from the 'yellow eel' to the silver phase of *Anguilla*. The physiological significance of such changes is not known or is imperfectly understood.

It has been assumed that the silvering of the eel is a 'breeding' dress. Of *Salmo* such an assumption—if less explicitly made—has been shown to be unfounded by recent work of Orton *et al.* (1938), Jones & Orton (1940) and Jones (1940). These authors have found that a substantial proportion of male salmon parr becomes sexually mature before the change to the silvery smolt dress.

The present communication records data resulting from experiments designed to explore the role of endocrine agencies in relation to the change to the silver so-called breeding dress of *Anguilla vulgaris* L. and *Salmo salar* L. In the course of these experiments it has become clear that serious misconceptions, to which it is desirable to draw attention, prevail with regard to sexual differentiation in the former species.

I. SILVERING OF *ANGUILLA*

(1) *Significance of the Syrski organ*

The accepted criterion of sex distinction is the *organ of Syrski*. In view of confusion regarding this structure, it may be recalled that Syrski's (1874) work antedated modern methods of histology and modern concepts of sex. Syrski inferred the existence of a sex distinction from the fact that silver eels may be divided into two classes: (*a*) larger ones with gonads containing recognizable oocytes, (*b*) smaller ones with 'lobed', i.e. more coarsely laminated, gonads in which no visible oocytes can be detected. Petersen (1894) emphasizes that the two groups are relatively discontinuous with respect to size.

Implicitly Syrski took for granted that the change from yellow to silver corresponds to the putting on of a breeding dress, i.e. denotes pending sexual maturity,

n assumption repeated explicitly by subsequent authors, e.g. Petersen (1894) imself.

The validity of the criteria used as a basis of sex distinction in *Anguilla* may be uestioned, especially because records of indisputable, i.e. sexually mature, males are xtremely few. A Syrski organ is by general admission a structure in which no ripe xual elements are as yet recognizable, i.e. it is an undifferentiated gonad. The line f reasoning followed by most, if not all, workers who have sexed eels would appear o be as follows:

(*a*) members of most species may be divided into two sexes;

(*b*) these may be distinguished by their gonads after sexual maturity;

(*c*) the eel becomes silver at the time of sex differentiation when two types may e distinguished;

(*d*) of these one type is indisputably female;

(*e*) so the other type is presumptively male.

In the absence of a detailed cytological study of the gonads of the eel over a long eriod this argument fails to carry conviction unless it is proved that the silvering f the eel represents the assumption of a breeding dress. The relative size discon- nuity emphasized by Petersen (1894) is irrelevant, since both growth and meta- orphosis are seasonal phenomena. In other words, there is necessarily at least ne difference of a year's growth between two eels that do not undergo metamorphosis n the same season. So if we assume that sex differentiation and metamorphosis ommonly occur about the same time, we should expect a more or less marked iscontinuity between smaller silver eels with an undifferentiated gonad and larger lver eels in which the gonad is recognizably of one or other sex.

In comparatively recent studies contemporary workers in eel fisheries, e.g. Tesch 1928) and Jespersen (1926), have evaluated the secondary sex characters of the eel vith no more certain guidance than the Syrski organ to indicate which individuals re males. Tesch (1928) divided eighty eels 20–25 cm. long into two groups. One roup was killed. All of these had Syrski gonads (i.e. were supposed to be males). The second group was kept in running water and fed on dead shrimps. Of these, wenty-one killed after one year's captivity also had Syrski gonads. The remaining els, twelve in number, proved after two years' captivity to be females (30–45 cm. n length) with 'true ovaries in which ova were developing'. Prof. Lancelot Hogben, n whose laboratory the present work was carried out, wrote to Dr Tesch asking vhether it was a correct inference that the so-called males had spermatozoa. Dr Tesch eplied that the males were recognized as such by their Syrski organs, and that these rgans, as is well known, do not contain ripe gametes.

What Tesch has therefore shown is that a Syrski organ may become an ovary n the normal course of events. This is not surprising, since the organ is an undifferen- ated gonad and there is not the slightest reason to question the validity of his data. rom his experiments Tesch draws the conclusion that the eel is a protandrous ermaphrodite. Since (*a*) *all* his animals presumptively had Syrski gonads at the tart, (*b*) *all* of those kept in captivity for two years were recognizably female in the nd, all that his observations definitely prove is that the Syrski organ is not a

sufficient criterion of maleness. These facts may be interpreted in three ways, any one of which is more plausible than the inference that the eel is a protandrous hermaphrodite:

(1) The eel (like *Cynips kollari*, *Rhodites rosae*, *Artemia* sp. and many other arthropods) is a perpetually parthenogenetic form of which functional males rarely turn up.

(2) The genetic mechanism of sex determination in eels is so nicely poised that (*a*) environmental changes which normally bias sex differentiation in favour of femaleness are all-important, (*b*) the production of a relatively minute proportion of mature males suffices to ensure fertilization in the localized breeding ground of the species.

(3) Metabolic differences which antedate visible sex differentiation of the gonads lead to choice of different habitats by eels of different sex.

Of these (2) appears the most likely, though there is yet no conclusive evidence that either of the others are wrong, and it is difficult to see that such proof could be obtained without further research on the habits of the eel in its own spawning ground. So far we have no information about the spawning habits of the eel.

After the present work was begun an account of experiments by Tuzet & Fontaine (1937) was published. In 1933 these authors recorded active spermatogenesis after injection of human pregnancy urine into three eels which were already in the silver condition. They recorded better results (five specimens) in 1937 after similar treatment of silver eels 36–40 cm. long kept in darkness. One of these shed spermatozoa into the water. All these eels were caught in Loire and the Marais de Brière. This work suggests that the absence of light favours maturation of the gonads of male eels.

(2) *Body size and gonad condition in yellow and silver eels*

For the purposes of the present work on the problem of silvering we shall, therefore, regard the organ of Syrski not as an index of maleness, but simply as an undifferentiated gonad. The correctness of this view has been checked in every case by microscopic sections. As will be seen from what follows no indisputably male eel (i.e. one with gonads showing spermatogenesis) has been encountered, nor has spermatogenesis been induced during the whole of these experiments up to date.

The investigation began with the collection of data concerning the degree of development and cytological condition of the gonads of both yellow and silver eels of various sizes. The eels used were caught in June 1937 from the River Dee at Aberdeen some two miles from its mouth.

The relation between body weight and gonad weight in twenty-five female eels is shown in Text-fig. 1, which shows that the weight of the gonads increases with the length of the eel. This appeared to be due solely to increase in the amount of fat deposited in these organs. The data show no significant difference with respect to relative gonad weight between silver and yellow eels of a given length. The gonads of all eels were sectioned and examined microscopically. The relevant findings were:

(*a*) The gonads of *all* eels over 30 cm. long were recognizably female. No detectable difference was found between the appearance of the oocytes present in the gonads of yellow and silver eels of this size.

(*b*) Smaller yellow eels had gonads containing no recognizable oocytes.

(*c*) One smaller silver eel had an undifferentiated gonad.

Thus the preliminary sample supplied no justification for associating the change from yellow to silver with differentiation of sex or maturation of gonads.

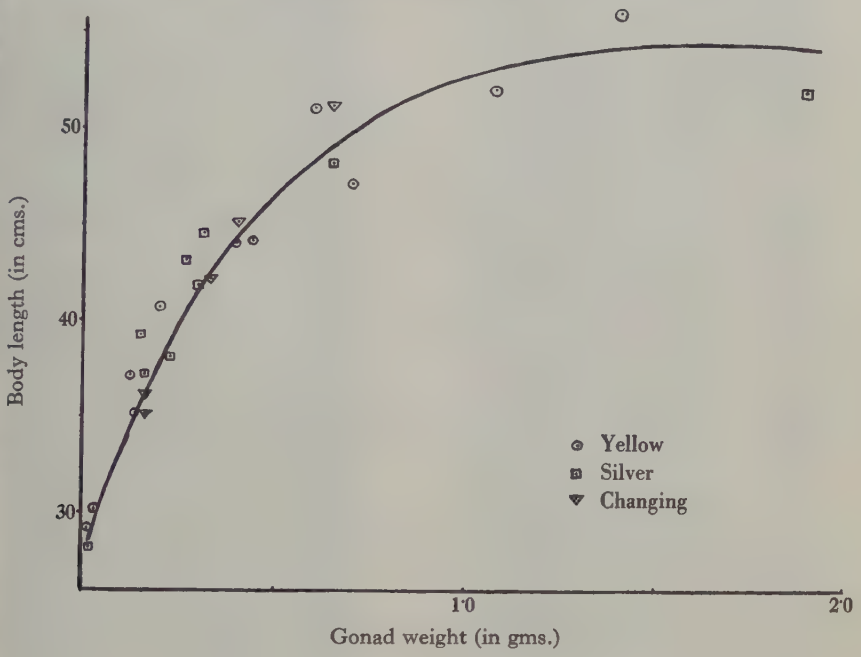


Fig. 1

(3) *Effect of injections of extracts on* Anguilla

Previous experiments on the effects of anterior lobe extracts, thyroid extracts and the urine of pregnant women had been carried out and were subsequently repeated. The activity of the urine and anterior lobe extracts used in all the experiments described was tested. Every extract and every specimen of urine gave a positive Hogben test (Crew, 1939; Landgrebe, 1939) with *Xenopus*. Each injection of thyroid and pituitary extracts represented the equivalent of 1 g. of fresh tissue. Each group of eels (six or twelve) was kept in a large black tank fitted with a closely fitting lid.

(*a*) The first experiment was started in 1937, when eighteen yellow eels 39–46 cm. long were divided into three groups of six. Each group of eels was kept in aerated tap water. The water was changed twice a week. The animals in the first group were injected weekly with an extract of ox anterior lobe of the pituitary. Those in the second group received a weekly injection of an extract of ox thyroid. The third

group were kept as controls. The experiment was continued for three months. The eels were then killed and examined. There was no difference between the three groups in the external appearance of either the animals or their gonads. Histological examination showed no difference between the gonads of the three groups. All of them were immature.

(*b*) After Tuzet & Fontaine (1937) had obtained positive results in experiments on the eel after injection of human pregnancy urine, it was necessary to repeat this preliminary experiment, using both pregnancy urine and anterior lobe extracts.

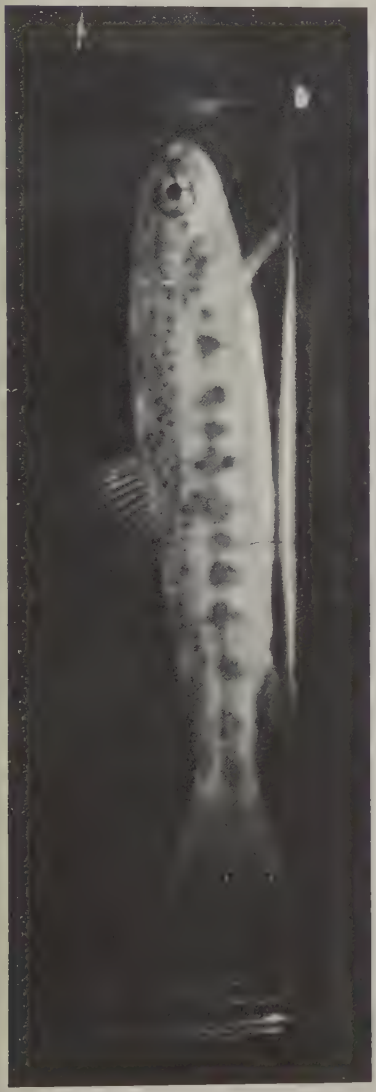
In a second experiment, undertaken in February 1938, eighteen yellow (44–48 cm. long) and eighteen silver (48–51 cm. long) eels were divided into three groups, each containing six yellow and six silver. The animals were kept under the same conditions as before. The eels of the first group were injected bi-weekly with an extract of ox anterior lobe of the pituitary. The eels in the second group were injected twice a week with 2 c.c. of pregnancy urine. The third group were kept as controls. The animals were killed after two months and no difference between the condition of the gonads in the two groups was detectable.

(*c*) A third experiment began in 1939. Four batches of twelve yellow eels (30–35 cm. long) were used. No silver eels of this size could be obtained at this time from the Dee. They were kept in running fresh water in darkness in black tanks fitted with lids. The animals in the first batch received an injection of extract of fresh pig anterior lobe tissue once a week. Those in the second batch were injected weekly with an extract of fresh ox anterior lobe tissue. The eels in the third batch were injected weekly with 2 c.c. of pregnancy urine. The fourth batch were kept as controls. The experiment was continued for ten weeks. The animals were then killed and examined. There was no observable difference between the batches either with respect to the external appearance of the eels or of their gonads. Histological examination showed that the gonads of all the animals were immature. The gonads of the experimental eels did not differ from those of the controls.

(*d*) Previous to the work recorded in this publication the author had found that the ovaries of *Bufo vulgaris* do not respond to injections of extracts similar to those used in the preceding experiments. However, the gonads of this toad can be matured and ovulation can occur after pituitary homeo implantation. A further attempt was made, therefore, to ripen the gonads of yellow eels by implanting freshly dissected eel pituitaries. Over a period of three weeks, eighteen glands were implanted into one animal. After a further six weeks, examination of the implantation site showed that most of the implanted glands had established themselves. Microscopical examination showed no change in the condition of the gonads.

(4) *Effect of injection of extracts on* Salmo salar

A similar experiment was then performed on salmon with the assistance of Dr C. A. Wingfield, who is carrying out experiments on the effect of temperature and of mineral constituents of the water on the rate of growth of this species (*S. salar*). Six salmon parr, obtained from Thurso in March 1939 as yearlings, were used in these experiments. They were divided into groups of two fish and set



(*a*)

(*b*)

Salmo salar. (*a*) Control. (*b*) After bi-weekly thyroid injection for two months.

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out in running fresh water in three small white sinks. The fish were fed daily with liver. Injections began when the animals were about 20 months old. The first group were injected twice weekly with the equivalent of 0·5 g. ox anterior lobe pituitary tissue. The second group were injected with an extract of ox thyroid equivalent to 0·5 g. fresh tissue twice a week, and the third group remained as controls.

All six fish had characteristic parr markings at the beginning of the experiment. After one month those fish injected with thyroid showed marked silveriness and those in the group injected with anterior lobe extract showed slight silveriness. After two months the fish injected with thyroid were quite silver and the parr markings had completely disappeared. The control fish had not changed in appearance (Pl. 1). Fish injected with anterior lobe tissue assumed a silver condition more slowly and in addition acquired the typical green coloration generally taken to be an indication of the onset of maturity. Both groups had completely lost their parr markings and were of the same appearance as smolts. After treatment the six fish were killed and weighed. Their gonads were also weighed before fixation in Bouin's fluid. The gonad body-weight ratio of the three groups did not differ significantly; and histological examination showed that gonads of all of them were immature. This confirms what the work of Orton and others has shown already. That is to say, the silvering of salmon is not of itself a sign of the onset of sexual maturity.

Crude extracts of anterior lobe tissue were used in this experiment and contained, among other autacoids, both gonadotropic and thyrotropic hormones. The silveriness which followed injection of this extract may have been due to stimulation of the animal's own thyroid.

(5) *Experimental silvering of the brown trout*

Systematists commonly recognize silver sea trout and brown river trout as varieties of the same species (*S. trutta*), though in fact relatively little is known about the life history of the former. Concerning the latter it is definitely known that no transition to a silvery condition accompanies sexual maturity or occurs at any stage of the life cycle in its fresh water habitat. During the summer of 1940 the writer was able to obtain a batch of brown river trout of the same hatching from Mr R. M. Neill of this laboratory.

Three groups of four fish 18 months old were kept together in a large aquarium in running fresh water. The fish in each group were distinguished by clipping the dorsal fin and the tail fin respectively in each of two groups. The fish in the first group were given bi-weekly injections of ox anterior lobe extract equivalent to 1 g. of fresh tissue. Those in the second group received thyroid injections equivalent to 1 g. of fresh tissue. The third group were kept as controls.

Within one month the trout which had thyroid injections appeared silvery and outwardly resembled 'sea trout' of similar size. Even after two months those fish injected with pituitary extracts did not differ in appearance from the controls. At the end of two months the trout were killed and the gonads examined. There was no difference with respect to the weight or appearance of the gonads between the three groups.

These results indicate (*a*) that there is no direct organic link between silveriness and sexual maturity; (*b*) that 'silvering' is brought about by direct action of the thyroid extract; (*c*) that the crude pituitary extract injected into the first group of fish was not sufficient to stimulate the animal's own thyroid. It is not surprising that greater stimulation would be necessary in the river trout than in salmon as silvering does not naturally occur in the former.

In view of the fragmentary state of our knowledge concerning the genetic relationship of the sea trout and the brown river trout, the experiments last recorded raise issues which would merit further enquiry before publication in circumstances other than the present. Owing to the uncertainty of enjoying further opportunities for immediate investigation, it is only possible to indicate some of the issues raised.

Since systematists do not agree among themselves about the definition of a species, the mere fact that the two types of trout are placed in the same specific category is unimportant from the experimental standpoint. These experiments seem to show that the difference between them is associated with functional activity of the thyroid gland. If we admit this conclusion several possibilities arise:

(1) Since the iodine content of sea water is very much higher than the iodine content of fresh water, we might first assume that any trout supplied with sufficient iodine would undergo silvering and that whether it becomes a sea trout is an accident of habitat, i.e. depends on whether or not it gets into an estuary.

(2) We may supplement the same initial assumption with the qualification that some stocks genetically differentiated from other stocks in this respect migrate down-stream as they approach maturity, so that only certain stocks get into the situation in which the thyroid is able to accumulate sufficient iodine to manufacture the threshold thyreoglobulin for silvering.

(3) We may reject the assumption common to (1) and (2) and assume that different trout stocks differ with respect to the iodine threshold of the thyroid gland. So that only those with a more sensitive thyroid are capable of becoming silver under natural conditions.

In other words, we may assume that the thyroid of some brown trout is incapable of discharging its active product owing to low pituitary activity—a possibility excluded by the negative effects of pituitary injection.

Of the three possibilities enumerated above, the third is excluded by the circumstance that brown trout are not found at sea. Furthermore, it does not seem likely that the one sample chosen for these experiments would be composed exclusively of individuals capable of becoming sea trout. We are left only with (1) and (2); and it would be a comparatively easy matter to exclude (1) by bringing the iodine concentration of fresh water in which brown trout are kept up to the level of what is found in sea water. Should the results prove to be negative, the second possibility stated above seems to be the correct one. If so, river trout and sea trout constitute two morphologically differentiated and discrete breeding units. So according to any intelligible definition of the word 'species' they are specifically distinct. Further work will show whether this is true.

SUMMARY

1. There is no satisfactory evidence for regarding the organ of Syrski as the male gonad of *Anguilla*.

2. Silvering of the eel, salmon and trout is independent of gonad development and cannot therefore be regarded as a 'breeding dress'.

3. Injection of either pregnancy urine, ox anterior lobe extracts, pig anterior lobe extracts and thyroid extracts, as also pituitary homeo-implantation, were all without effect on the gonads of eels treated in this way.

4. Ox anterior lobe extracts produced premature smoltification in *Salmo salar*, had no visible effect on *S. trutta* or on *Anguilla vulgaris*, and did not affect the gonads in either species.

5. Injection of thyroid extracts produced silvering in salmon and brown trout but had no effect on the eel.

6. After two months' treatment with thyroid extract brown trout are externally indistinguishable from sea trout.

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A STUDY OF THE RELATIVE TOXICITY OF ANIONS, WITH *POLYCELIS NIGRA* AS TEST ANIMAL

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(With Six Text-figures)

INTRODUCTION

In the study of the physiological effects of electrolytes the attention of scientists has generally centred on the action of cations, and the effects of anions have not been investigated to the same extent. Richet (1881), an early student of the toxicity of salts to aquatic animals, noticed that nitrates are more toxic than chlorides, but little attention was paid to the physiological effects of anions, apart from those whose high toxicity interested the pharmacologist, until Hofmeister (1888) showed that neutral sodium salts differed greatly in their power of precipitating egg albumen. According to the limiting molar concentrations at which the different salts effected immediate precipitation, certain anions could be arranged in a definite series, which has come to be known as the 'Hofmeister' or 'lyotropic' series. Later it was found that the Hofmeister series was applicable to some variety of phenomena in colloid chemistry, and the interest of biologists was aroused. Lillie (1906) concluded that according to their inhibitory effect on ciliary motion in the gills of *Mytilus* the anions can be arranged in the order $SCN > I > Br$, $NO_3 > SO_4 > Cl$, acetate, and obtained (1910) a somewhat similar series for the toxicity of sodium salts to unfertilized *Arbacia* eggs. Similarly, Schwartz (1907) found that the return to irritability of the sartorius muscle of *Rana esculenta*, after immersion in sugar solutions, was favoured by sodium salts according to the order $CNS > I > Br > NO_3 >$ acetate $> SO_4 >$ citrate. Raber (1920), employing the well-known technique devised by Osterhout (1918), studied the effect of a number of sodium salts on the permeability of *Laminaria*, and obtained an anion series closely resembling those obtained by Hofmeister. One of the most intensive of such studies is that of Kaho (1921), whose investigation included series of sodium, potassium and ammonium salts, *Tradescantia* and red cabbage being the biological materials. Here again the characteristic anion sequence appeared, though the position of many ions varied somewhat according to the concentration and the cation employed.

The results so far reviewed have drawn considerable criticism. Kaho's results have been criticized by Heilbrunn (1928, p. 164) on the grounds that Kaho compared the effect of equinormal solutions, and that the solutions of tartrate, citrate and sulphate were much weaker osmotically than the solutions of monovalent salts,

which were apparently hypertonic. Gray (1928, p. 97) shows that the series obtained by Lillie for the effect of anions on ciliary motion in the *Mytilus* gill is erroneous, and that the facts observed really illustrate the order in which the sodium salts with different anions influence the stability of the tissue when calcium and magnesium are absent. Loeb (1922) was one of the severest critics of the Hofmeister series, and appears to have decided that the series was an illusion; that the different effects produced by different sodium salts were simply due to changes in the hydrogen ion concentration, and that when the *p*H is kept constant the effect of different ions is determined solely by their valency. This is a somewhat unworkable hypothesis, and it is useless to deny that certain anions, in their effect on living organisms at least, have a specific action independent of their valency; even Loeb himself (1912, p. 155) has drawn attention to the specific action of the cyanide ion. Furthermore, Gellhorn (1932, 1933) has shown that the Hofmeister series holds for the excitability of striated muscle, and the restoration of automaticity of heart muscle, even when the *p*H of the solutions is kept constant at 6·6.

Mathews (1904) made a study of the relative toxicity of seventeen anions to the eggs of *Fundulus*, and Raber followed the paper previously cited (Raber, 1920) with further studies (1921 *a*, *b*, *c*) on the effect of anions on *Laminaria*. Ellis (1937, p. 428) supplies a certain amount of information regarding the effect of a number of sodium salts to fish, but there appears to be no really comprehensive study of the relative toxicity of anions to a living animal; probably this is because physiologists have been so preoccupied with the Hofmeister series. In the present study, with the planarian *Polycelis nigra* (Müller), twenty-seven anions have been included.

EXPERIMENTAL DETAILS

Sodium salts are generally used in the study of the physiological effects of anions, because they are all soluble, and because sodium has such a low degree of toxicity that, except in the case of salts of extremely low toxicity (e.g. NaCl), the effect of the cation can be neglected. Potassium and ammonium, the other bases with long series of soluble salts, are decidedly more toxic than sodium, while calcium, another innocuous cation, forms comparatively few salts of sufficient solubility.

In a previous study (Jones, 1940), dealing with the action of cations on *Polycelis*, the writer has discussed some of the methods employed for the estimation of the toxicity of substances to aquatic animals, and has shown that the best criterion to adopt is the threshold of toxicity. This, in the case of *Polycelis*, can be taken as the approximate concentration at which the animal survives immersion in the solution for 48 hr. The same criterion has been adopted in the present investigation; with each substance tested a survival curve was worked out covering times up to 60–70 hr., and the approximate concentration corresponding to a 48 hr. survival time thus determined. In early experiments five animals were used at each concentration tested, their individual survival times observed, and the means plotted. This procedure proved very tedious, and later it was found reasonably satisfactory, and very much more convenient, to place nine animals in each solution and observe the time

at which the fifth animal died; that is, the survival times recorded were median, not mean, values. In cases where most of the animals in a solution died during the night the experiment was repeated. Water distilled in glass was used for all solutions. For other experimental details see previous papers by the writer on the toxicity of salts to *Polycelis* (Jones, 1937, 1940). A representative series of the survival curves obtained is given in Fig. 1.

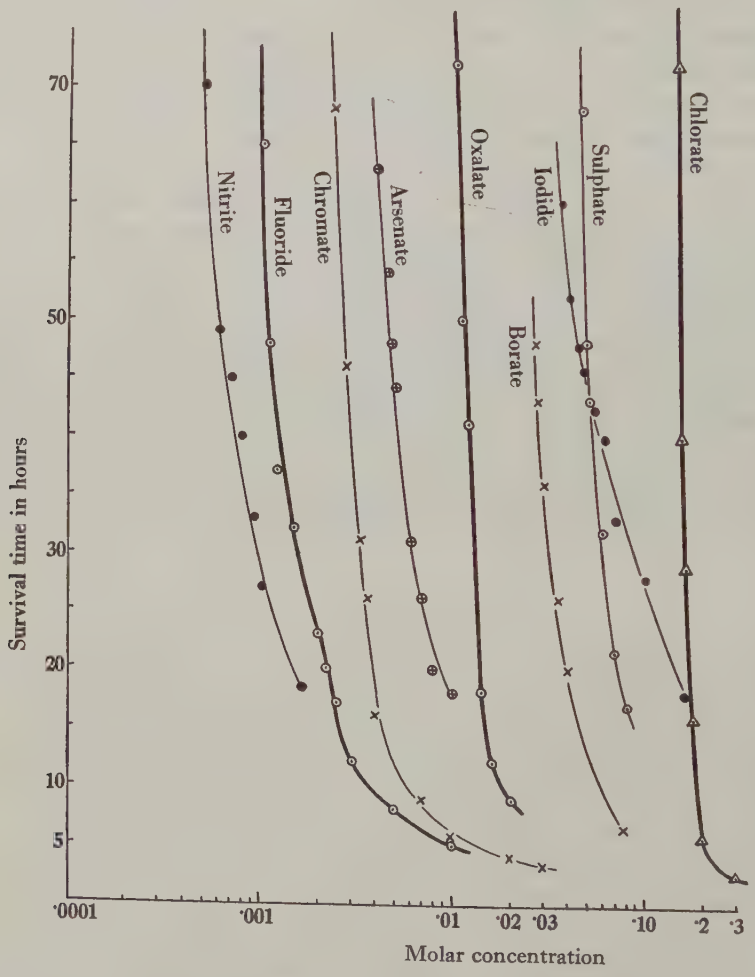

Fig. 1. *Polycelis nigra.* Representative series of survival curves for anions. For composition of solutions see Table 1. Each plotted point represents the median survival time for nine animals in 30 c.c. of solution. The concentration scale is logarithmic. Temp. 15–18° C.

With most of the substances tested the threshold of toxicity was found to be reasonably well defined. For example, in the case of sodium oxalate, 50 hr. after the commencement of the experiment all the animals had died at concentrations of 0·014 *M* and above, eight out of nine had died at 0·012 *M*, five were dead in the 0·011 *M* solution, and at 0·010 all were alive. In some cases, notably sodium iodide,

the survival curve has a comparatively gentle slope, and the threshold of toxicity is less well defined.

In many cases the preparation of approximately neutral solutions demanded special attention. Many anions form very weak acids, and, as sodium is a strong base, their sodium salts form highly alkaline solutions. The stock phosphate solution consisted of a 0·3 *M* solution of sodium phosphate (Na_2HPO_4) to which was added

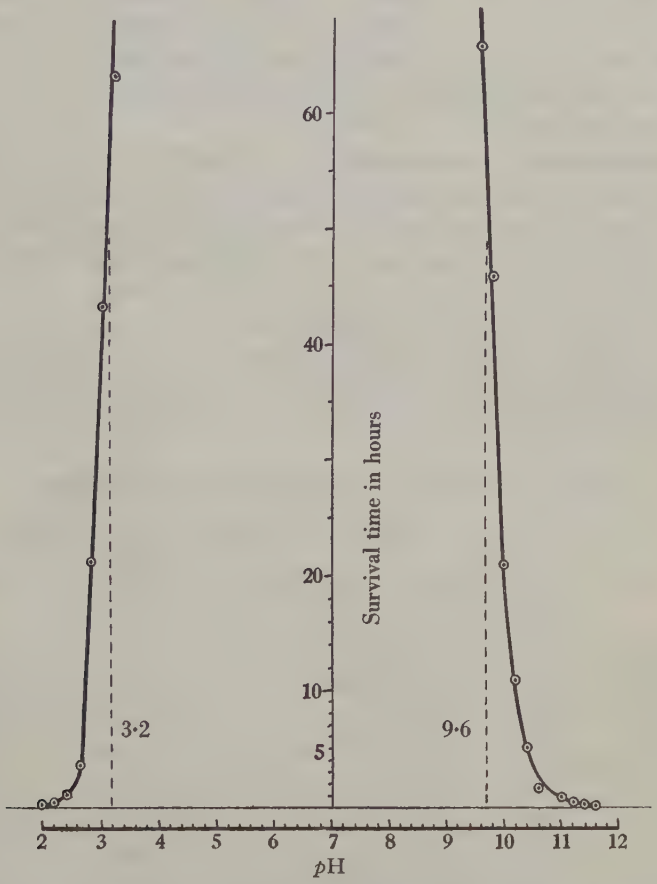

Fig. 2. Survival curves giving the survival times of *Polycelis* at high and low hydrogen-ion concentrations, and indicating the comparative toxicity of hydrogen and hydroxyl ions. Temp. 15–18° C.

sufficient of a 0·3 *M* solution of phosphoric acid to make an approximately neutral solution. Similarly the citrate solution was compounded from sodium citrate and citric acid; the chromate solution from sodium chromate and chromic acid. The borate stock solution consisted of a 0·3 *M* solution of boric acid to which was added a very small quantity of a 0·075 *M* solution of sodium tetraborate.

The preparation of neutral carbonate solutions proved impossible, except at great dilutions. Sodium carbonate solutions are highly alkaline, and even sodium bicarbonate, though an 'acid' salt, gives solutions of high *p*H. By adding about 4 %

(molar proportion) of HCl to a concentrated solution of $NaHCO_3$ the *p*H can be brought down to about 8·0, but attempts to reduce the *p*H still further result in effervescence of carbon dioxide. The experiments were therefore run at a *p*H of about 8·0, but the solutions proved very unstable, becoming more alkaline on standing, and had to be renewed every 12 hr. Some difficulty was also experienced with sodium sulphite; freshly prepared solutions are alkaline, but if they are made neutral by addition of sulphuric acid they become very acid after standing for a few hours, and the animals die as a result. Later it was found that pure solutions of sodium sulphite become nearly neutral after standing for some hours, and the final experiments were run with sulphite solutions allowed to stand for 12 hr. before the animals were put into them.

The survival times of *Polycelis* at different hydrogen ion concentrations are given in Fig. 2, and it will be seen that on the basis of a 48 hr. survival time *Polycelis* will tolerate a *p*H range of about 3·2–9·6. The alkaline limb of the curve lies decidedly nearer the neutrality line than the acid limb, and thus hydroxyl ions appear to be more toxic than hydrogen ions. *Polycelis* survives a solution of *p*H 3·2 for over 48 hr.; a solution containing an equivalent concentration of hydroxyl ions (*p*H 10·8) is fatal in about 65 min. Some physiologists appear to attribute no toxic action to the hydroxyl ion, and regard the lethal effect of highly alkaline solutions as due simply to a deficiency of hydrogen ions.

Table 1. *Thresholds of toxicity for* Polycelis nigra

Temp. 15–18° C.

Anion	Composition of solutions	Molar concentration anion	*p*H
Chloride	NaCl	0·19	6·0
Chlorate	$NaClO_3$	0·15	6·0
Acetate	CH_3COONa	0·15	7·2
Bromide	NaBr	0·14	6·4
Carbonate	*$NaHCO_3$ + HCl	0·085	8·0
Tartrate	$Na_2(CHOH.COO)_2$	0·065	7·0
Thiosulphate	$Na_2S_2O_3$	0·053	7·0
Sulphate	Na_2SO_4	0·048	6·6
Sulphite	Na_2SO_3	0·048	7·2
Iodide	NaI	0·044	6·6
Nitrate	$NaNO_3$	0·043	6·4
Phosphate	$Na_2HPO_4 + H_3PO_4$	0·026	7·2
Borate	$H_3BO_3 + Na_2B_4O_7$	0·026	7·4
Bromate	$NaBrO_3$	0·020	6·4
Citrate	$Na_3C_6H_5O_7 + H_3C_6H_5O_7$	0·015	7·2
Thiocyanate	NaCNS	0·012	6·6
Oxalate	$Na_2(COO)_2$	0·011	6·4
Arsenate	$NaHAsO_4$	0·0048	7·8
Chromate	$Na_2CrO_4 + CrO_3$	0·0028	6·8
Iodate	$NaIO_3$	0·0013	6·6
Fluoride	NaF	0·0011	6·6
Ferrocyanide	$Na_4Fe(CN)_6$	0·0008	6·6
Nitroprusside	*$Na_2Fe(CN)_5.NO$	0·0008	4·8
Nitrite	$NaNO_2$	0·0006	6·6
Cyanide	*NaCN + HCl	0·0006	6·8
Sulphide	*Na_2S + HCl	0·00045	6·8
Hydroxide	*NaOH	0·00004	9·6

* Solutions renewed every 12 hr.

The *p*H of sodium hydroxide solutions down to 11·0 can be calculated from the dilutions, taking the dissociation constant of water as 10^{-14}. Over the range 9·4–11·0 it was necessary to prepare the solutions by a trial and error method; 500 c.c. volumes were employed and the *p*H determined colorimetrically, the indicators used being thymol blue, phenolphthalein and phenol-thymol-phthalein. The solutions of *p*H 9·4–10·0 were somewhat unstable and had to be renewed every 12 hr.

The results are summarized in Table 1, which gives for each anion the approximate molar concentration corresponding to a median survival time of 48 hr. It will be appreciated that no account is taken of degree of ionization, and thus the expression 'molar concentration anion' strictly speaking means the molar concentration of the acid radical in question. This, in most cases, is the molar concentration of the sodium salt. In the case of solutions with two components only the acid radical in question is taken into account. Thus a molar phosphate solution, containing Na_2HPO_4 and H_3PO_4, would be one containing 95·02 g. PO_4/l., a molar carbonate solution one containing 60 g. CO_3/l.

DISCUSSION

The original Hofmeister series expresses the effects of anions (of sodium salts) on the salting out, or precipitation, of egg albumen in neutral or weakly acid solutions, and is as follows:

$$\text{citrate} > \text{tartrate} > SO_4 > \text{acetate} > Cl > NO_3 > ClO_3.$$

If we arrange the same ions according to their toxicity to *Polycelis* we obtain the sequence

$$\text{citrate} > NO_3 > SO_4 > \text{tartrate} > \text{acetate},\ ClO_3 > Cl.$$

Some measure of agreement is evident, the main difference between the two series being in the position of the nitrate ion. The writer does not propose to discuss at length the degree to which the anion sequence given by Table 1 agrees with the series obtained by Hofmeister and other physiologists and biochemists; and it may be remarked that a perusal of the large number of different anion series to be found in the literature on the physiological and biochemical effects of anions inevitably leads to the conclusion that the Hofmeister series is a decidedly elastic arrangement. Furthermore, in a critical examination of the applicability of the Hofmeister series to the relative toxicity of anions to a living animal (which the present investigation does not pretend to be), we must be prepared to make considerable allowance for the permeability factor, a factor which does not intrude when dealing with the inanimate systems studied by Hofmeister and his co-workers. Krogh (1939) has shown that anions vary very greatly in the speed with which they pass into the body of an aquatic animal; in experiments with the 'wool-handed' crab, *Eriocheir sinensis*, it was found that Cl, Br and CNS ions are actively absorbed, NO_3 is not absorbed but diffuses into the body rapidly, I is absorbed slowly and SO_4 apparently not at all. Experiments with *Carassius auratus* showed that Br and Cl are actively absorbed, that I diffuses in slowly, while no penetration of NO_3 or CNS could be detected.

A comparison of Table I with the corresponding table for cations (Jones, 1940) indicates that anions, for the most part, are decidedly less toxic than cations. The most toxic anion (OH) is inferior in effect to Cd, Cu, Au, Ag and Hg, the last being 40 times as toxic as OH and 600 times as toxic as CN. In some cases similarity of composition appears to be associated with similarity in degree of toxicity, thus S_2O_3, SO_4 and SO_3 occupy adjacent positions. On the other hand, NO_2 is over 70 times more toxic than NO_3.

In the case of sodium chloride it is uncertain whether the toxicity of the salt is due to the cation, the anion or both, and it is probable that the lethal effect of high concentrations of NaCl are mainly due to osmotic pressure. In some instances the sodium ion appears to have a definite toxic effect; thus Lillie (1904) states that

Fig. 3. Sketch of three *Polycelis* after 10 min. immersion in a sodium hydroxide solution of *p*H 11·4.

NaCl has an almost immediate destructive effect on the cilia of the *Arenicola* larva which is not equalled by the chlorides of other metals.

It is probably correct to state that the precise physiological effect of many anions is not understood. The toxicity of oxalates is said to be due to their property of forming an extremely insoluble salt with calcium, thus removing from solution an element essential to the life of the organism (Martindale & Westcott, 1920, p. 74). Similarly, Mathews (1939) states that the high toxicity of fluorides is probably due to their power of removing magnesium and calcium from ionic solution. In pharmacology sodium nitrite and amyl nitrite are recognized as powerful vaso-dilators, lessening arterial tension and reducing the blood pressure (Martindale & Westcott, 1920, pp. 146, 707). This information obviously is not very helpful in the case of *Polycelis*. Sulphides, like cyanides, are stated to impede or inhibit protoplasmic oxidation (Mitchell, 1938, p. 650), and it is generally recognized that strong alkalis dissolve proteins and bring about the rapid necrosis and destruction of animal tissues. The effect of sodium hydroxide solutions on *Polycelis* is striking; solutions of *p*H 12–14, as might be expected, bring about an almost immediate

destruction of the animal. Solutions of *p*H 10·6–12·0 effect a rapid coagulation of the mucus covering the body, and the animal becomes enclosed in a white, tenacious covering which resembles frost or dried soap; this fixes the animal to the bottom of the dish and despite convulsive muscular effort it is rendered incapable of moving away. Two or more animals in close proximity will become enclosed in the same envelope (see Fig. 3). The coagulation of the mucus is followed by the rapid disintegration of the animal.

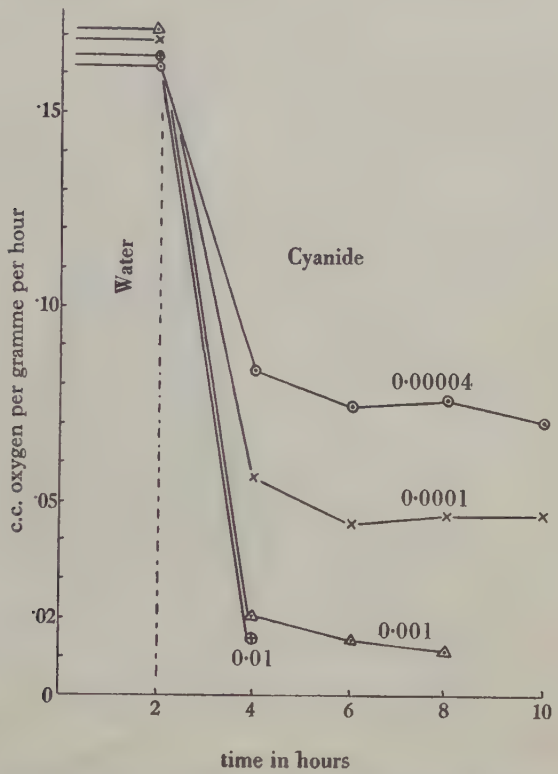

Fig. 4. The effect of sodium cyanide on the respiration rate of *Polycelis*. Each plotted point gives the average respiration rate in c.c. O_2 (N.T.P.) per g. per hr. for the preceding 2 hr. For the first 2 hr. the animals were in glass-distilled water, for the subsequent times in NaCN solutions of the molar concentration indicated. At each concentration 100 animals were used. Temp. 18° C. *p*H 6·8–7·2.

Edmunds & Gunn (1936, p. 648) state that ferrocyanides and other double cyanides are in most cases harmless. This hardly seems the case with the present test animal, but it may be noted that while sodium ferrocyanide is almost as toxic as sodium cyanide on a molar concentration basis, a molar solution of the former contains six times as much cyanogen as the latter. The depressing action of hydrocyanic acid and the cyanides upon protoplasmic oxidation has been demonstrated for a considerable variety of animal types. In mammals poisoned by cyanide the venous blood exhibits the scarlet colour of arterial blood (Edmunds & Gunn, 1936, p. 648), and at the opposite end of the evolutionary scale cyanides inhibit the

luminescence of *Noctiluca*, a process dependant upon protoplasmic oxidation (Harvey, 1917). The effect may not be universal; Lund (1918) has claimed that the respiration rate of *Paramecium* is not depressed by cyanides, but his results have been severely criticized by Hyman (1919). *Polycelis* survives a 0·0006 *M* solution of NaCN for about 48 hr.; according to Alexander *et al.* (1935, p. 108) a 0·000015 *M* cyanide solution causes rainbow trout to float upside down in 11 min. Thus the

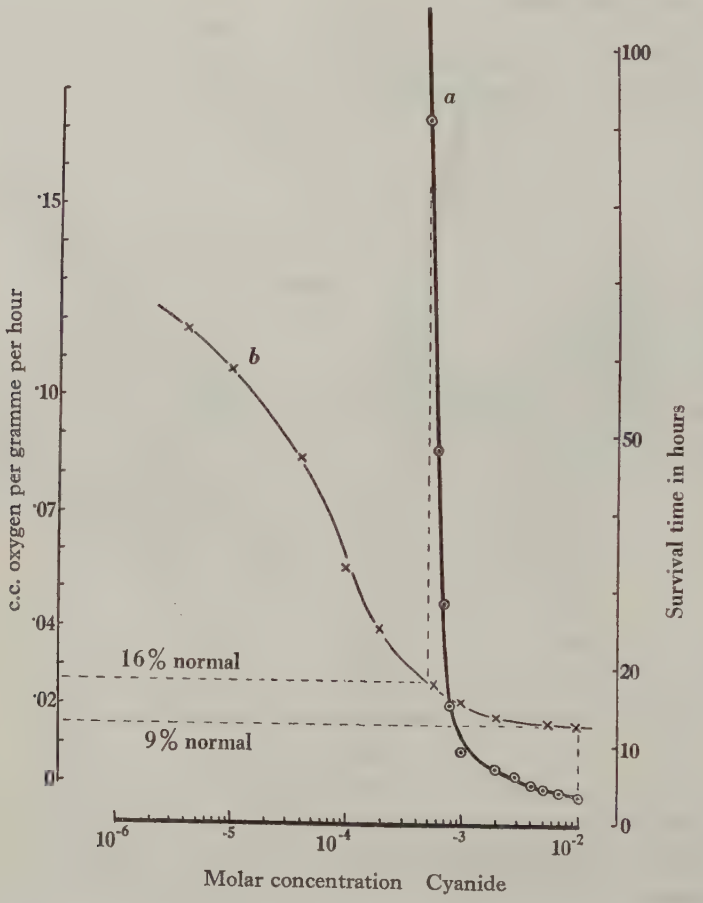

Fig. 5. *a*, survival curve for *Polycelis* in sodium cyanide. *b*, average respiration rate for 2 hr. immersion in sodium cyanide of molar concentration indicated by the same scale. The cyanide solutions were brought to a *p*H of 6·8–7·2 by the addition of HCl. Temp. 18° C.

effect of cyanide upon *Polycelis* is comparatively slow. The normal oxygen consumption of this animal does not differ greatly from that of active fish; a number of determinations were made by the simple technique described by Krogh (1916, p. 50), and seven experiments at 18° C. gave the average result 0·165 c.c. O_2/g./hr. This compares with 0·22 c.c./g./hr. for the trout (at 14·7° C.) and 0·07 at 14·7° C. for the goldfish. These figures are taken from data collected by Heilbrunn (1937, p. 199). In these experiments 100 *Polycelis* were placed in a 70 c.c. bottle with a

perforated stopper, which was filled with glass-distilled water of known oxygen content, the bottle placed in a thermostat, and the oxygen content determined 2 hr. later (Winkler method). The animals were weighed after the experiment, each being dried with filter paper. The effect of various concentrations of NaCN was then tried, the result being given in Fig. 4. This result is very similar to that obtained with *Planaria dorotocephala* by Hyman (1919), but in this investigation, and a similar one by Allen (1919), no particular study seems to have been made of the

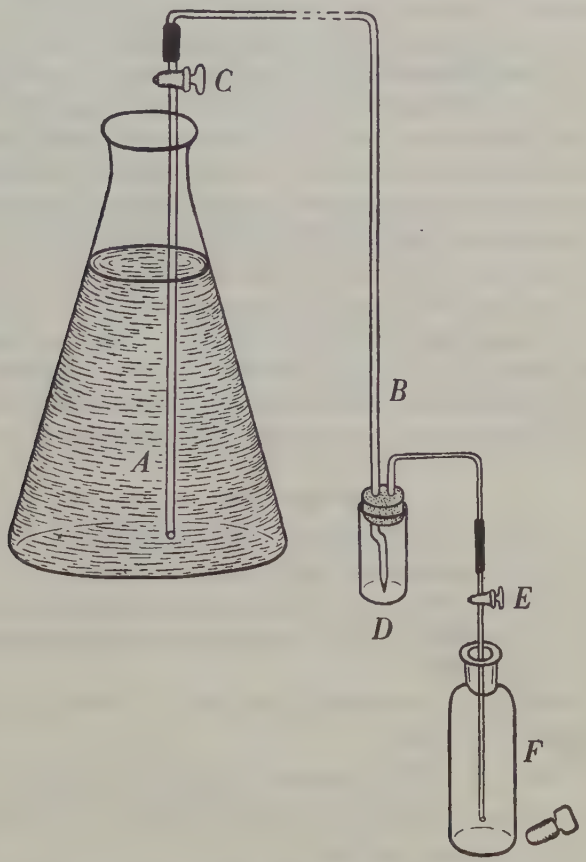

Fig. 6. Apparatus for determining the survival time of *Polycelis* in boiled water. Explanation in text.

relation between the survival time of the animal and the degree to which the respiration rate is depressed. In Fig. 5 the survival curve for *Polycelis* in NaCN is drawn, and against this is plotted the average oxygen consumption in c.c./g./hr. for 2 hr. immersion in solutions of a molar concentration indicated by the same horizontal scale. It will be seen that concentrations of cyanide much lower than the 'threshold of toxicity' depress the rate of respiration to a marked extent, but that the survival time is prolonged or indefinite as long as the respiration rate is not less than about 16% of the normal value. If the respiration rate is reduced further

the survival time shortens rapidly, and at 0·01 *M*, when the respiration rate is 9% or normal, the animal lives less than 4 hr.

The comparative slowness of the effect of cyanide upon *Polycelis* is probably connected with the animal's capability of surviving for a considerable time in water of very low oxygen content. The apparatus sketched in Fig. 6 was employed to determine the survival time of *Polycelis* in water that has been boiled and cooled out of contact with the air. About a litre of water was placed in flask *A*, the siphon *AB* was filled, placed in position, and the tap *C* closed. The water was then boiled for an hour, the level being maintained as necessary by the addition of water kept boiling in a second flask. Immediately boiling was stopped the surface of the water was covered with a layer of liquid paraffin about an inch deep. When the water had cooled fifty *Polycelis* were placed in the tube *D*, the taps *C* and *E* opened, and about 700 c.c. of the boiled water run through; this overflowed through the sampling bottle *F*. Immediately the flow of water was stopped the sample bottle was removed and the oxygen content of the water determined. In a typical experiment the animals crawled about for the first 2 hr., and then became inactive. The average survival time was about 40 hr., the fifty animals weighed 0·3 g., the capacity of *D* and the tubes connected to it was 20 c.c., the oxygen content of the water was 0·9 c.c. O_2/l., and the average amount of oxygen available for the animals was therefore 0·0015 c.c./g./hr. This, of course, does not include the reserve of free oxygen present in the bodies of the animals, and in the slime that covers them.

SUMMARY

A brief review is given of existing knowledge regarding the physiological effects of anions, and literature dealing with their relative toxicity.

The degree of toxicity of twenty-seven anions to *Polycelis nigra* (Müller) has been assessed, by determining in each case the molar concentration the animal survives for 48 hr. at 15–18° C.

On this basis their order of increasing toxicity is as follows; commas separate ions of similar degree of toxicity:

$Cl < ClO_3$, acetate, $Br < CO_3 <$ tartrate $< S_2O_3 < SO_4$, $SO_3 < I$, $NO_3 < PO_4$, $BO_3 < BrO_3 <$ citrate $<$ CNS, $C_2O_4 < AsO_4 < CrO_3 < IO_3 < F < Fe(CN)_6$, $Fe(CN)_5NO < NO_2$, $CN < S < OH$.

Generally speaking anions are very much less toxic than cations. Even the most toxic anion (OH) is far less toxic than ionic copper, silver or gold.

The respiration rate of *Polycelis* is heavily depressed by cyanide, but the survival time is three days or longer, as long as the respiration rate is not less than about 16% of the normal value. With further depression the survival time shortens rapidly, and at 9% normal is under 4 hr.

The normal respiration rate of *Polycelis nigra* is 0·165 c.c. O_2/g./hr. This is not very much less than that of the trout. *Polycelis* is considerably the more resistant to cyanide. This is probably connected with its capability of surviving very many hours in water containing a very reduced supply of oxygen.

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THE BIOLOGY AND BEHAVIOUR OF *PTINUS TECTUS* BOIE. (COLEOPTERA, PTINIDAE), A PEST OF STORED PRODUCTS

I. THE DAILY RHYTHM OF LOCOMOTORY ACTIVITY, ESPECIALLY IN RELATION TO LIGHT AND TEMPERATURE

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(*Received* 8 *May* 1941)

(With Eight Text-figures)

GENERAL INTRODUCTION

Ptinus tectus Boie. is a spider beetle which was first described by Boieldieu (1856). It was not recorded in Britain until the beginning of this century, and it did not attract much notice as a pest of stored products until after 1920 (Hinton, 1941). By 1937 it had been recorded in a greater variety of foods in railway warehouses than any other insect pest (Hayhurst, 1937). In 1940, the Grain Pest Survey found that this species had become a primary pest of stored grain and was apparently able to attack undamaged grain (Munro, 1940). *P. tectus* comes to us from the temperate climate of Tasmania and the adjacent parts of Australia, and it does not require high temperatures for successful colonization. Thus in this country the species has spread and established itself rapidly, and it may become still more important.

Most of the work so far published on *P. tectus* has been concerned with its foods and the duration of its life cycle, number of eggs laid and so on, under various conditions. Much of this work is slight and some of it has been found in this laboratory to be inaccurate and misleading. Apart from this, there appear to be only one publication on cold resistance (Mansbridge, 1936), and two doctorate theses which include some experimental work on temperature preference (Fahmy, 1931; Deal, 1939).

In 1939 *P. tectus* was suggested by Prof. J. W. Munro, of the Imperial College of Science and Technology, as a species suitable for concentrated investigation. The work was made possible by the facilities available in this department at Birmingham and has been assisted and encouraged at all stages by Prof. H. Munro Fox, F.R.S. Contact has been maintained with the work of the D.S.I.R. Pest Infestation Laboratory at Slough through Dr O. W. Richards, to whom we are indebted for the benefit of his wide experience and critical judgment.

[1] The records which had to be made in 8 hr. shifts throughout the day were made equally by all three authors, but all the other experimental work and the planning were done by E. W. B. in consultation with D. L. G.

I. INTRODUCTION TO DIURNAL RHYTHM

It has been shown that the common cockroach walks about much more in the night than in the daytime and that this rhythm of locomotory activity can be reversed by a reversal of the rhythm of light and darkness (Szymanski, 1914; Gunn, 1940; Mellanby, 1940). It seems likely that responses of animals to physical stimuli will sometimes depend on whether they are in the active or the inactive phase of such a diurnal rhythm. It has been shown, for example, that certain copepods react differently to gravity according to the time of day (Esterley, 1917). This work on the rhythm of *Ptinus* has therefore been carried out as a preliminary to other work on the behaviour of the species. The simplest method of estimating activity was adopted; every 15 min. the animals were observed and the number walking about was counted. The variations in activity so recorded were correlated with various conditions of illumination and temperature. As will be seen, it has been found that there is a daily variation of activity of *Ptinus* and that this rhythm can be influenced by both of these factors.

II. MATERIAL AND METHODS

The animals used in the following experiments were cultured at 25° C. in glass jars of about 2½ litres capacity, on a mixture of "Artox" wholemeal flour with 5% of dried brewer's yeast. In each jar there was a glass tube of 1 in. diameter containing wet cotton wool as a source of drinking water. By this method a relative humidity of about 70–80% was maintained.

All the experiments were conducted in the standard choice chambers described by Gunn & Kennedy (1936). Except in chambers containing flour, there was a false floor consisting of a platform of finely perforated zinc which was supported by dishes containing a suitable mixture of sulphuric acid and water to control the humidity. The zinc was cut to fit the chamber but could not be fitted accurately enough to prevent the animals from getting between it and the chamber wall into the acid. This difficulty was overcome by fitting around the edge of the false floor a length of tightly coiled spiral spring ('extensible curtain rod'), with a copper wire core for rigidity. In the experiments with flour, the chamber was used without the zinc platform, and with about 5 mm. of wholemeal flour on the floor. The flour was first conditioned to subtend a relative humidity of about 60%, and was smoothed down before the animals were put in. In the experiments without flour, no food was provided.

Most of the experiments to be described were carried out at three different humidities (34, 60 and 95% R.H.), controlled by sulphuric acid-water mixtures (Landolt-Börnstein, 1905). At the end of each experiment, the humidity was checked by means of an Edney paper hygrometer calibrated against acid-water mixtures (Gunn & Kennedy, 1936).

Some of the experiments were done in a constant temperature room at 25° C. and others in two rooms where there was a diurnal cycle of temperature. Both of these rooms were centrally heated and the heating was cut off at the end of the after-

noon, so that the temperature fell until about six o'clock in the morning. Temperatures were recorded on a thermograph and checked by thermometer readings every quarter of an hour. Details of temperature fluctuations are given in the descriptions of the experiments concerned.

Three principal sources of light were used. The first was a tungsten filament lamp giving an intensity of 20–30 m.c. The second was a neon glow lamp giving an intensity which was difficult to define but was usually between 0·3 and 1·2 m.c.: a grease spot photometer was used for the measurement. Since the observations of activity were made visually, it was necessary to have some light during the darker period and the neon light was used for this purpose, being the faintest light which was adequate. In preliminary experiments *Ptinus* was subjected to light of 20–30 m.c. after a long period in darkness; activity was high at first and then gradually fell off. On the other hand, when prolonged darkness was followed by neon light, activity was not high at first and did not fall off afterwards. It is reasonable to assume, therefore, that continuous illumination from a neon lamp may be considered as equivalent to darkness in the interpretation of the results. If this be accepted, the method may find more universal application in diurnal rhythm investigations, since the alternative method of using an aktograph is slow in yielding sufficient results to be statistically significant and is not easy to use with animals as small as *Ptinus* (2–3 mg.). The third kind of light used was natural daylight on a bench under a north window, and in this case a neon lamp was also in use day and night.

In the constant temperature room, experiments in constant light were conducted on a table immediately below a high ceiling light which gave a light intensity at table level of 20–30 m.c. For the experiments in alternating light and darkness, a corner of the constant temperature room was curtained off. As a 'day' light each dish was illuminated by a 25 W. lamp at a distance of 36 cm. which was shaded so as to bear only on the one dish and which gave the standard light (20–30 m.c.) at the level of the zinc floor. Experiments in continuous 'darkness' were conducted in a second curtained-off part of the constant temperature room and, as in the alternating light experiments, a basic lighting (by neon lamps) was maintained.

Light intensities were measured by a 'New Avo' light meter made by the Automatic Coil Winder and Electrical Equipment Co., Ltd. In the experiments under natural conditions, readings were taken every quarter of an hour as long as the daylight was bright enough to give a reading at all.

Five animals were used in each dish. This was a compromise between using a number small enough to be observed accurately at one moment and one large enough to give statistically reliable results quickly.

In recording activity three states of behaviour were recognized (Gunn & Pielou, 1940). First, animals which were inactive, i.e. which showed no movements whatsoever; second, animals which were active in such a way as to move to another part of the chamber; and third, an intermediate type of behaviour which is termed 'virtual inactivity' and which includes movements of the antennae, turning round, copulation (or pseudo-copulation), struggling on the back or any other movement which has no translocatory effect.

Observations were made by a 'cross-section method', in which the observer first finds the approximate position of all the animals (sometimes a lengthy process in the 'dark'), and then, after looking away again, notes, in terms of the above mentioned categories, the behaviour of the animals at a random instant.

Readings were taken every quarter of an hour throughout the whole 24 hr. of each day and the majority of the experiments lasted four or five days. Consequently the work of taking readings was divided between three observers, each doing an 8 hr. shift. This meant that each observer covered the same hours each day, but the experiments were conducted in two groups with an interval of several weeks between them, and in the second group the shifts were reversed relative to the light conditions to which the animals were subjected. The readings of the three observers were standardized by preliminary readings made simultaneously. After the experiments, the results recorded by the three observers were also examined statistically to try to detect any individual trend which might have influenced them, and reasonable consistency seems to have been attained.

In the final treatment of the results, for a number of the experiments, separate consideration was given to activity and to activity plus virtual inactivity. It was found, however, that the conclusions which it was possible to draw were not affected by the inclusion of virtual inactivity and therefore, in order to simplify presentation, only locomotory activity is considered below. In neon light virtual inactivity was often difficult to distinguish reliably from complete inactivity.

Before setting up any experiment, all animals were given an opportunity of making up any deficiency of water by keeping them for 1–2 hr. in a vessel containing wet cotton-wool. In the first batch of experiments the animals were introduced at 12.00 Greenwich Mean Time and readings began 2 hr. later. In the other experiments an interval of 14 hr. was allowed to elapse before readings were started. During this interval, the animals were more active because they had been recently handled; this high activity was irrelevant to the rhythm and was therefore ignored. In each of the following graphs, the activity is expressed as a percentage of the maximum possible, and the period covered is about 5 days.

III. EXPERIMENTS AT 25° C.

(*a*) *Alternating light and darkness*

In Fig. 1 are shown the results of experiments at three relative humidities (34, 60 and 95 % R.H., respectively), in alternating light and darkness (light from 06.00 to 18.00 G.M.T., 'darkness' from 18.00 to 06.00 G.M.T.), with animals which had been subjected to the same conditions for ten days previously. Fig. 2*a* shows the three experiments combined. It will be seen that *Ptinus* showed a well marked diurnal rhythm. The greatest activity occurred immediately after the change from light to darkness, and there was a second much smaller peak of activity about dawn.

In most of the experiments, an attempt was made to decrease the disturbing effect of the sudden change of light intensity by making the change slowly by means of a rheostat arranged as a potentiometer. There seemed to be little effect of having

this protracted dusk or dawn (which lasted about 27 min.), instead of a sudden switching over. As will be seen below, in the experiments with naturally varying light and temperature (§ IV), there were also two peaks, a large one at about dusk and a smaller one at dawn.

(*b*) *Continuous light*

Observations were made in continuous light on animals which had been cultured in continuous light for some months at a constant temperature of 25° C. A typical result is shown in Fig. 4 for an experiment at 60% R.H. Fig. 2*b* shows the combined

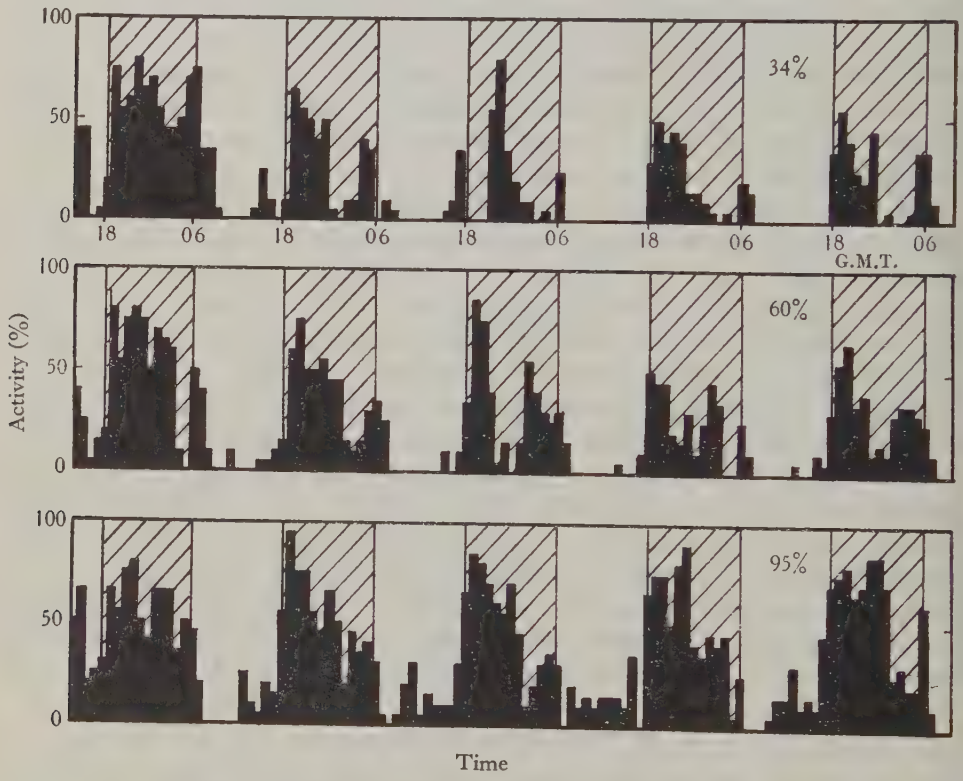

Fig. 1. Activity of *Ptinus tectus* Boie. in alternating light and darkness at three different humidities. Animals previously kept in similar alternation.

results of two other experiments. It will be seen that there was no inherent rhythm, comparable to that described above. In another experiment there was some evidence of a diurnal rhythm, but there was reason to believe that the culture from which the animals had been taken had not been exposed to light of constant intensity. A bench light had been in daily use nearby about midday. This suggests the possibility of inducing a rhythm by alternating light of two intensities, of which the lower need not be so dim as our neon lighting; there is also the possibility that a rhythm can be induced by means of a brighter light used for a shorter period than our 12 hours (cf. Kalmus, 1940).

(*c*) *Persistence of the rhythm in continuous light*

Simultaneously with the above experiments in alternating light and darkness, records were made of the activity of animals from the same culture in constant light. Fig. 2*c* shows the combined results of three experiments at 34, 60 and 95 % R.H. respectively. It will be seen that the rhythm shown in Fig. 2*a* is very definitely carried over into constant light, but begins to lose its identity in a few days.

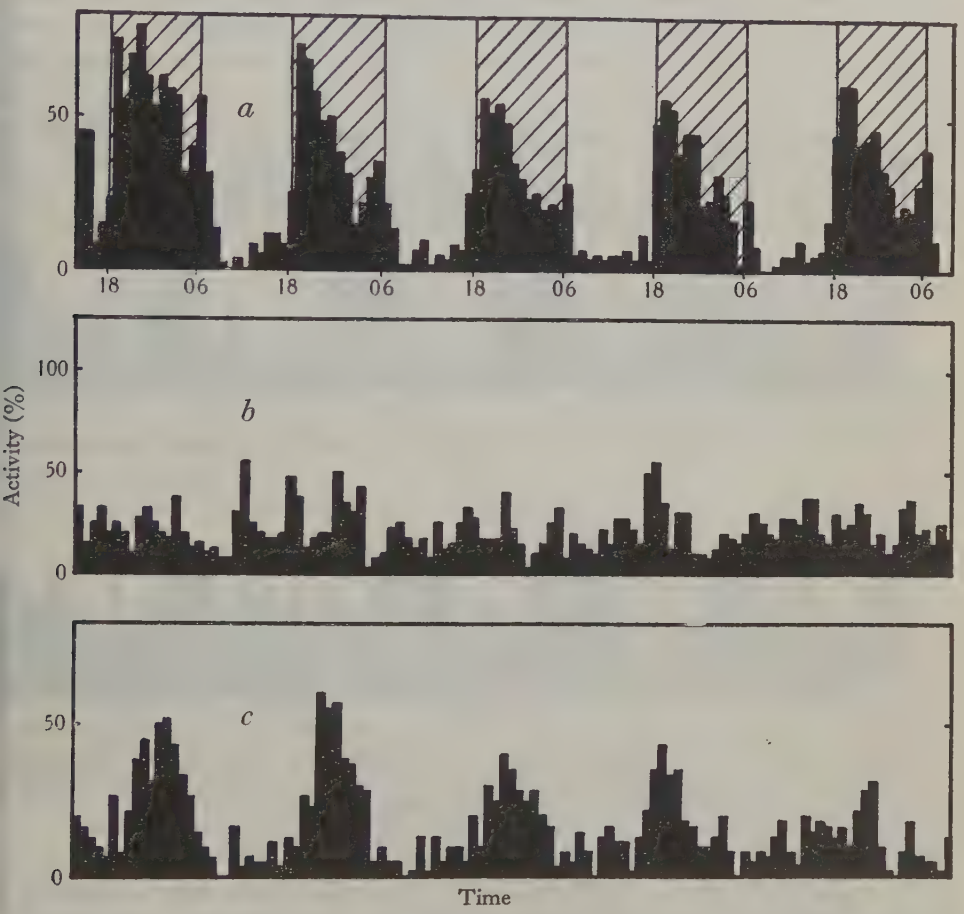

Fig. 2. Activity of *Ptinus*. Combined results of (*a*) the three experiments in alternating light and darkness in Fig. 1, (*b*) *two* experiments in constant light with animals previously in constant light, (*c*) three experiments in constant light using animals from previously alternating light and darkness.

(*d*) *Reversal of the rhythm*

For these experiments, animals were taken from the same culture as the animals used in the experiments described in §§ III (*a*) and III (*c*); they were suddenly transferred to reversed conditions of illumination (light from 18.00 to 06.00 and

'darkness' from 06.00 to 18.00 G.M.T.), kept in these conditions for 3 weeks and then tested. Fig. 3 *a* shows the results. Here again, the greater part of the activity occurred in the dark period. In one experiment at 95 % R.H., activity started earlier in the day than might be inferred from Fig. 2*a*, but the maximum nevertheless occurred in the first few hours of darkness. Thus, the rhythm of locomotory activity which *P. tectus* shows in alternating light and darkness can be controlled irrespective of the time of day.

The effect of transferring 'reversed' animals to continuous darkness was to abolish the rhythm immediately. At a constant relative humidity of 34 % long periods of very low activity were recorded, while at 95 % R.H. continuous high

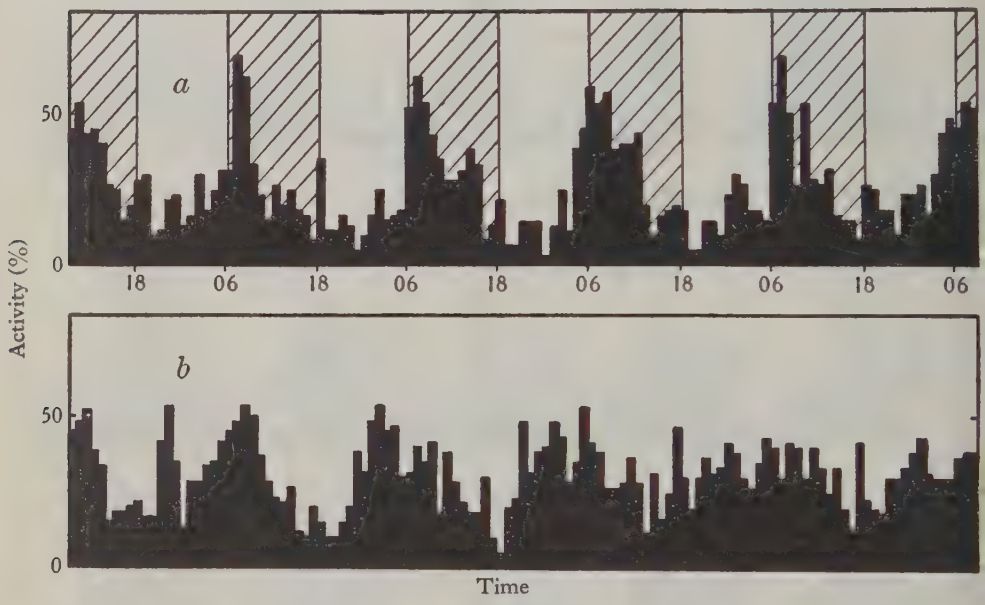

Fig. 3. Activity of *Ptinus*. Combined results of three experiments at different relative humidities with animals which had been kept in conditions of 'reversed' alternation of light and darkness: (*a*) in similar alternating conditions, (*b*) in continuous light.

activity occurred. There was, however, reason to believe that temperature conditions in this experiment were not quite constant and that they opposed the effect of the previous light rhythm. The results must therefore be treated with caution.

The effect of transferring 'reversed' animals to continuous light was that the rhythm continued for several days, but soon became blurred (Fig. 3 *b*). It is noticeable, however, that the time of maximum activity quickly advanced from 06.00 to about 24.00 G.M.T. This was the middle of the light period for the 'reversed' rhythm. This change might have been due to the persistence of an effect of the normal rhythm of three weeks before, inadequately overcome by the 3 weeks of reversed light conditions (cf. Lutz, 1932).

(*e*) *Initiation of a rhythm*

Animals which had been kept in continuous light were tested for three days, and they showed no diurnal rhythm of activity (Fig. 4). Then the normal alternation of darkness and artificial light was started. During the first period of darkness, the animals showed no increase of activity, but during the subsequent light period,

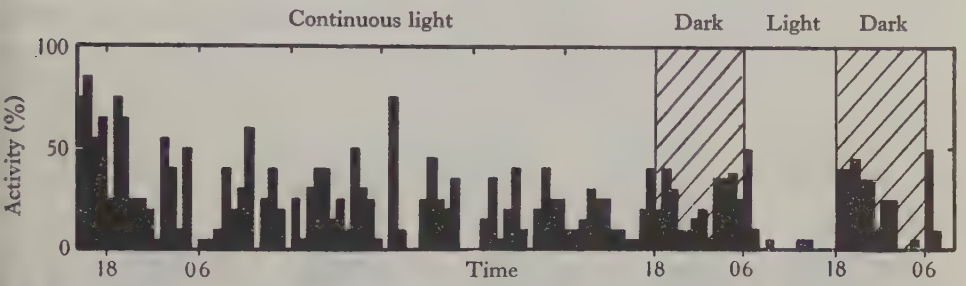

Fig. 4. Activity of *Ptinus* in an experiment at 60 % R.H. in which animals from continuous light were transferred to alternating light after three days.

activity was negligibly small. Then activity recurred in the next period of darkness (Fig. 4). There is insufficient evidence from our experiments to show what period must elapse before the rhythm is strong enough to be continued in constant conditions of illumination.

IV. EXPERIMENTS IN NATURALLY VARYING TEMPERATURE AND LIGHT

Fig. 5*b* shows the combined results obtained in experiments at three humidities, in conditions approximating to those in which *Ptinus* is found, namely, with fluctuating temperature and natural light intensities. The maximum and minimum temperatures recorded were 20·4 and 10·9° C. After 3 days the experimental chambers were transferred to continuous light and constant temperature (25° C.).

It will be seen that *Ptinus* again showed greatest locomotory activity in the dark and that this rhythm was maintained for at least a couple of days, though with decreased activity, under constant conditions of light and temperature. On comparing Fig. 5*a* (constant temperature) with Fig. 5*b* (fluctuating and lower temperature) it will be seen that the activity recorded was higher in the latter; further, the transfer of animals from alternating to continuous light produced a considerable reduction in activity when the temperature simultaneously changed from fluctuating to constant (Fig. 5*b*), but not so great a reduction when the temperature was constant (cf. Figs. 2*a*, 2*c*). One possible explanation of these facts is that temperature and temperature fluctuations have a previously unsuspected importance in the activity rhythm. Accordingly, experiments were carried out in constant light and fluctuating temperature.

V. EXPERIMENTS IN CONSTANT LIGHT WITH NATURALLY VARYING TEMPERATURE

The animals used had been kept in constant light with naturally varying tem perature for at least 3 weeks previously. The culture jar in which they lived wa supported during this time on shock-absorbent material to guard against diurna variation in gross vibration. The temperature during the experiment varied betwee 17·2 and 23·0° C. Three relative humidities were used and, during part of the experi ment, simultaneous observations were made of the changes shown by Edne

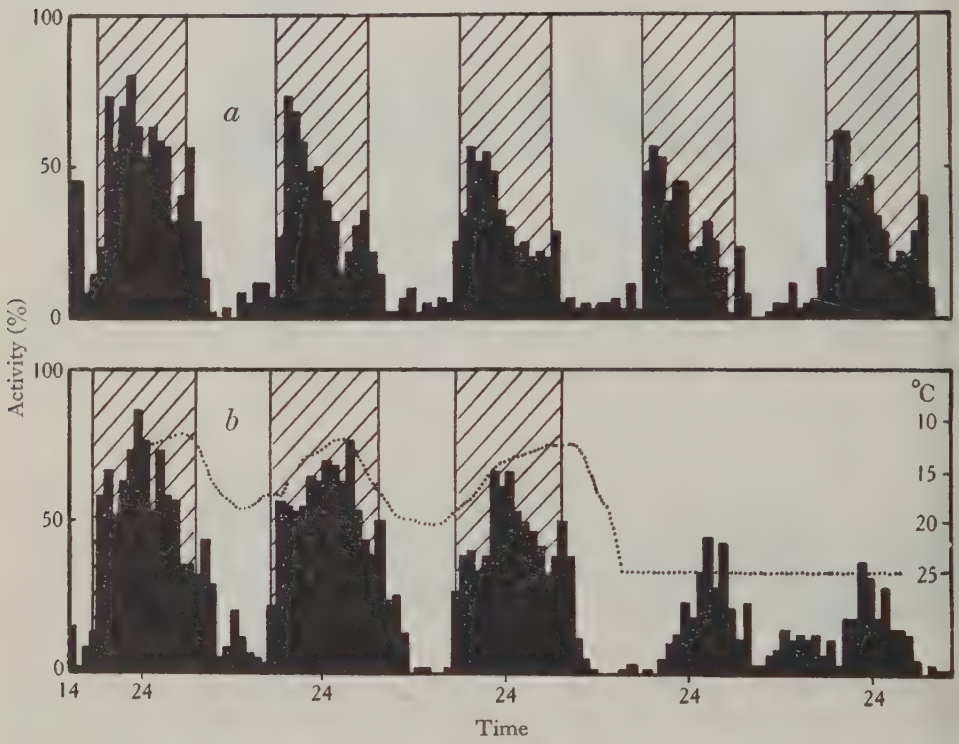

Fig. 5. Combined results of three experiments (*a*) at 25° C. and in artificially alternating light ar darkness, (*b*) with fluctuating temperature and natural day and night illumination. After three da the animals were transferred to 25° C. and constant light. The daily temperature fluctuations a shown; note that the temperature scale is inverted.

hygrometers enclosed in chambers identical with the experimental chambers. C course, this method cannot give an exact record of the lag in the maintenance of th required humidity during temperature changes, because there is some lag in th hygrometers themselves, but it does give an indication of the order of magnitud of any humidity changes taking place. The maximum diurnal fluctuation recorde at 34 was $1\frac{1}{4}$% R.H. At 60 and 95% R.H. the maxima were $1\frac{1}{2}$ and $1\frac{3}{4}$ R.H. r spectively. After 3 days the experimental chambers were removed to consta temperature at 25° C.

It will be seen from Figs. 6 and 7 that though the light conditions were constant, *Ptinus* still showed a definite rhythmically recurring activity and that greatest activity occurred at the time of falling and low temperature. In constant light and temperature, the rhythm was clearly continued in two of the chambers but not so obviously in the third (60 % R.H.) where there was a greater spread of activity during the first day (Fig. 6). There was no slowing up or accelerating effect of the higher temperature on the rhythm (cf. Kalmus, 1934) but the experiments were not protracted enough to reach any definite conclusions in this direction.

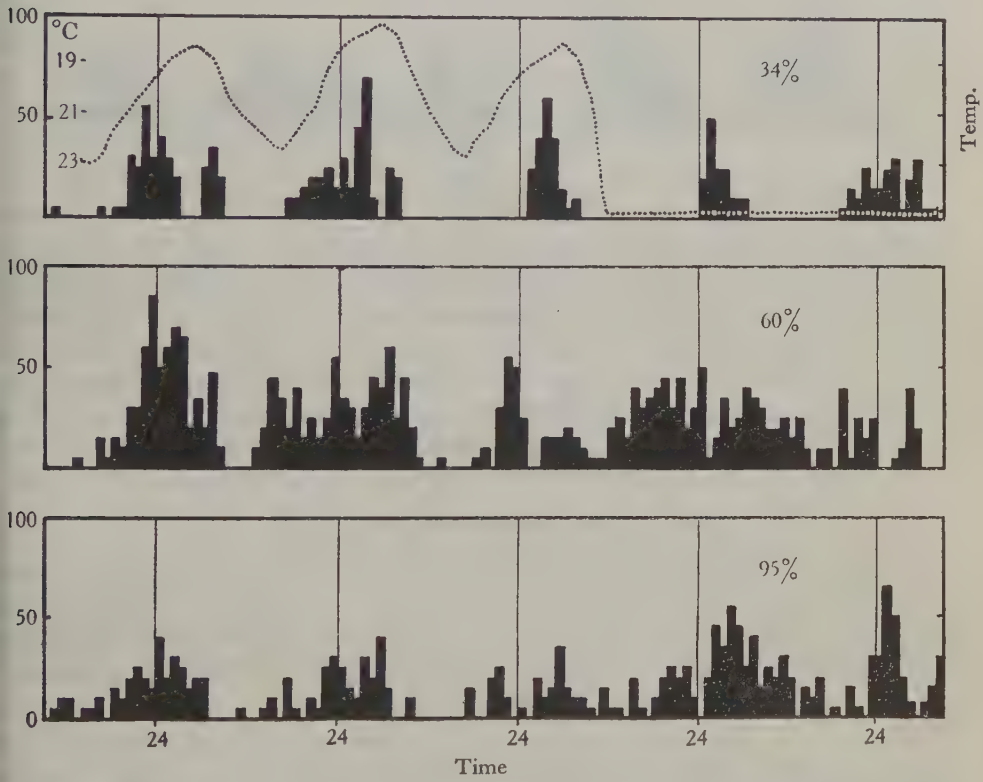

Fig. 6. Activity of *Ptinus* in three experiments with animals previously in constant light and fluctuating temperature, kept under similar conditions for three days and then transferred to constant light and 25° C.

As indicated above, simultaneously with temperature changes, there occurred small changes in relative humidity. It may be asked whether the rhythm exhibited under the conditions of the above experiment can be ascribed to temperature changes or to humidity changes or to both. The humidity changes were small and took place slowly and continuously. Animals are known, however, to respond to humidity changes of quite small extent. Thompson (1938) recorded sensitivity to humidity differences of 0·05 % R.H. per cm. in *Culex fatigans*. Pielou & Gunn (1940) describe an intense reaction near saturation point for *Tenebrio molitor* (see also

Kennedy, 1937). It is important, however, to realize that these reactions took plac at certain critical relative humidities and large differences over other parts of th scale had little or no effect on the animal's reactions. Moreover, in these experi ments, the insects experienced the two slightly different humidities within the ver short period taken to fly or walk 20 cm., while in our experiments on the rhythm o *Ptinus*, the slight humidity change took place over periods of hours. If, therefore

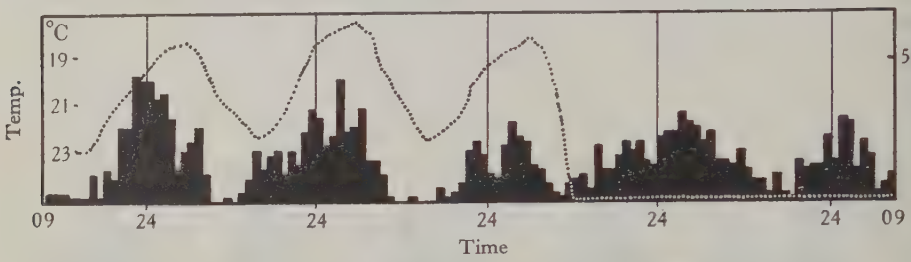

Fig. 7. Activity of *Ptinus*. Combined result from the three experiments of Fig. 6.

we are to draw the conclusion from the above that *Ptinus* might be reacting t humidity changes, we have to postulate at least three relative humidities at whic *Ptinus* shows an intense reaction. Such behaviour has not been recorded for an insect. Further, unpublished results from experiments on the humidity preferenc of *P. tectus* have shown that though the beetle does react to humidity differences the small fluctuations which occurred in the rhythm experiments can have had, a the most, a reinforcing effect on the more important factor of temperature.

VI. EXPERIMENTS WITH FLOUR

It is not proposed here to discuss at any length the results of those experiment in which the animals were provided with 60% R.H. Artox flour. In general the bore out the conclusions drawn above, with the difference that the total activity wa very much less than in the case of the animals without food. Sometimes, even in th

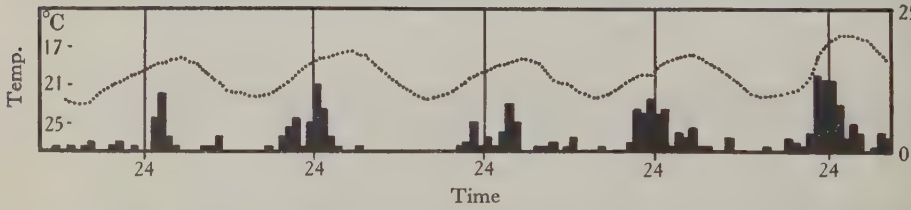

Fig. 8. Total activity of *Ptinus* from experiments on flour, in constant light and fluctuating temperature.

dark period, activity was negligible. Fig. 8 shows the totals, for example, of th activity in two chambers in constant light and fluctuating temperature over a perio of 5 days. The apparatus was supported throughout on shock-absorbent spong rubber. As will be seen, there was little activity, but such activity as there wa occurred when the temperature was falling.

DISCUSSION

Various authors have put forward evidence of an inherent rhythm more or less independent of external environmental changes. For example, the time memory shown by bees and ants comes in this category (von Stein-Beling, 1935). The greater part, however, of the work published on diurnal rhythms has pointed to daily fluctuations in light intensity as being the important factor in determining the distribution of activity. Thus, evidence has been brought forward to show that the cycle of light and darkness determines the active period of the cricket, *Gryllus assimilis* (Lutz, 1932), the vole, *Microtus* (Davis, 1933), the stick insect, *Dixippus morosus* (Kalmus, 1938), the deer-mouse, *Peromyscus* (Johnson, 1939), the cockroach, *Blatta orientalis* (Gunn, 1940; Mellanby, 1940) and the axolotl, *Amblystoma tigrinum* (Kalmus, 1940). There is thus no doubt that light is an important factor in controlling rhythmical activity in many animals, but this does not mean that light is always the only effective factor, or even the dominant one. It is possible that, in the field, activity is often controlled by a number of factors acting simultaneously, and this may be the explanation of the results obtained by Mellanby (1939) for the bed-bug. In this connexion Piéron (1937) has indicated that in some cases at least light may be acting as a clue to more important environmental changes.

Necheles (1927) has claimed that the activity of certain nocturnal insects is dependent on humidity and has suggested that such insects hide in the daytime in order to conserve moisture; this claim was based on little evidence, however, and has been criticized by Gunn & Cosway (1938). The importance of humidity as a factor in diurnal rhythms has also been stressed by Picard (1912) and Piéron (1937), but their evidence is slight.

The role of temperature as a factor controlling diurnal rhythm has been somewhat neglected. Bodenheimer & Klein (1930) have shown that the activity of the ant, *Messor semirufus*, in the field is directly dependent on the floor temperature. During the hotter months of the year, this ant emerges from its nest when the temperature is low, even though this occurs during the hours of darkness. During the cold season, activity occurs during the hours when the temperature is high. There is, however, no evidence that this rhythm would be carried over into constant conditions. Scott (1936) found a rhythm of pupal emergence controlled by temperature in *Ephestia kuhniella*. The period of maximum emergence coincides with a fall in temperature. We have found that, over the range presented (17–23° C.), *Ptinus tectus* is active during the cooler part of the day. It must be remembered that the criterion of activity used in these experiments is locomotory activity as distinct from speed of movement or energy expenditure. The effects of temperature on frequency of locomotion and rate of energy expenditure can be quite different (Nicholson, 1934; Fraenkel & Gunn, 1940) and there is thus no difficulty in believing that a fall of temperature may increase locomotory activity. The question of whether, over a lower temperature range, *Ptinus* would show increased activity with a *rise* of temperature awaits further experiment, but preliminary experiments made by Dr Hopf in this Department suggest that this is not unlikely.

In conclusion it is suggested that temperature (and possibly humidity, too), may play a greater part in controlling diurnal rhythm than has hitherto been realized and that further research is needed along these lines. The small variation in temperature (6° C.) which is needed to set up a rhythm in *P. tectus* indicates the importance of strict temperature control. Thus, the fluctuation of 1–2° C. in Lutz's (1932) experiments may explain the curious reversion to a normal rhythm shown by two specimens of *Gryllus assimilis*, after four days in constant darkness, though they had previously exhibited a reversed rhythm.

SUMMARY

In alternating light and darkness at 25° C., *Ptinus tectus* shows a diurnal rhythm of locomotory activity with maximum activity occurring in the dark period. The rhythm is continued for a few days in continuous light.

In continuous light, no inherent 24 hr. rhythm is apparent, but in subsequent alternating light and darkness, within 1 day, activity becomes practically confined to the dark period.

The rhythm can be reversed by reversing the hours of light and darkness and the reversed rhythm is similarly continued in continuous light.

In conditions of alternating light and darkness with fluctuating temperature (10–20° C. with low temperature in the dark periods) *Ptinus* shows greater activity than at 25° C., and maximum activity occurs in the cold, dark period.

In constant light and daily fluctuating temperature (17–23° C.), the period of greatest activity occurs when the temperature is falling. After transfer to constant temperature, this period still occurs at the same time of day for a few days.

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